Rh2 AND ITS AGLYCONE aPPD IN COMBINATION THERAPY WITH DOCETAXEL – PRE-CLINICAL EVALUATION IN A PC-3 HUMAN PROSTATE XENOGRAFT MODEL

by

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ABSTRACT

PURPOSE: The purpose of this thesis is to establish the therapeutic potential of ginsenoside Rh2, a 20(S)-protopanaxadiol type and its aglycone aPPD, two gastrointestinal metabolites of *Panax Ginseng* C.A. Meyer, for use in advanced prostate cancer. This thesis assesses (i) the pharmacokinetics, biodistribution and efficacy of Rh2 and aPPD as single agents, and (ii) the combination of Rh2 or aPPD with docetaxel with the goal of optimizing their therapeutic potential.

METHODS: The solubility and stability of Rh2 and aPPD in ethanol was determined and an oral dosage formulation was prepared. Rh2 and aPPD levels in blood and tissues following administration to nu/nu nude mice were determined by LC/MS. Their efficacy as single agents and in combination with docetaxel was determined in a PC-3 human prostate cancer xenograft model. Serum levels of AST, ALT, and creatinine were measured for toxicity. Tumor analysis for apoptotic indices and Ki-67 was performed. *In vitro*, PC-3, LNCaP, DU145, and C4-2 prostate cancer cells were treated with a combination of Rh2 or aPPD with docetaxel, according to the constant ratio combination design.

RESULTS: The solubility of Rh2 and aPPD in ethanol was 130 and 68.4 mg/ml respectively. *In vitro,* Rh2+docetaxel and aPPD+docetaxel both showed synergy (CI < 1) against PC-3 and DU145 cells. *In vivo*, Rh2 and aPPD exhibited a C_{max} of 19.0 ± 2.0 and 3.9 ± 1.4 μg/ml, respectively, while no toxicity was observed. They engendered a significant delay in PC-3 tumor growth, an increase in apoptotic index, and a decrease in Ki-67 levels. Docetaxel alone inhibited tumor growth by 84% but could not induce tumor regression while Rh2+docetaxel and

aPPD+docetaxel caused established PC-3 tumors to regress from their initial size by 15 and 27% respectively.

CONCLUSIONS: We have shown that Rh2 and aPPD are stable compounds that can be formulated for oral gavage. Pharmacokinetic studies demonstrate their ability to be absorbed following oral administration. Rh2 and aPPD can be combined with docetaxel to yield synergistic inhibition of cancer cell growth *in vitro*. Their *in vivo* combinations with docetaxel in a PC-3 xenograft model induce significant tumor regression compared to docetaxel dosed alone.

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LIST OF ABBREVIATIONS

5-FU:	5-fluorouracil
ABC:	ATP-binding cassette
ABCP:	ABC transporter in placenta
AD:	Androgen dependent
AI:	Androgen independent
ALT:	Alanine aminotransferase
ALT:	Alternative lengthening of telomeres
ANOVA:	Analysis of Variance
APC:	Adenomatous Polyposis Coli
AR:	Androgen receptor
AST:	Aspartate aminotransferase
a.u.c.:	Area under the curve obtained from a compound peak eluting from the HPLC
AUC:	Area under the plasma concentration versus time curve
BCRP:	Breast cancer resistance protein
BER:	Base excision repair
CAM:	Complementary and Alternative Medicine
CCAM:	Cell-cell adhesion molecules
CDK:	Cyclin-dependent kinase
CI:	Combination index
Cl_{oral}:	Apparent oral clearance

C_{max}:	Peak Concentration
C.V.:	Coefficient of variation
CYP:	Cytochrome P450
DAB:	3,3'-Diaminobenzidine
DCC:	Deleted in Colorectal Carcinoma
DHT:	5α-dihydrotestosterone
DMEM:	Dulbecco's Modified Eagle's Medium
DRE:	Digital rectal exam
DRI:	Dose Reduction Index
EBV:	Epstein-Barr virus
ECM:	Extracellular matrix
ECVAM:	European Centre for the Validation of Alternative Methods
EGCG:	Epigallaocatechin-3-gallate
EMT:	Epithelial–mesenchymal transition
FDA:	U.S. Food and Drug Administration
FGF 1/2:	Fibroblast growth factor
GI:	Gastro-intestinal
GF:	growth factors
GS:	Growth signals
GST:	Glutathione-S-transferase
HBV:	Hepatitis B virus

HGPIN: High-grade prostatic intraepithelial neoplasia

HIV: Human immunodeficiency virus

HPLC: High performance liquid chromatography

HPV: Human papilloma virus

HTLV: Human T-cell lymphotropic virus

IC: Inhibitory concentration

IND: Investigational New Drug

I.V.: Intravenous

KSV: Kaposi sarcoma virus

LC/MS: High performance liquid chromatography tandem mass spectrometry

LD_{50}: Median lethal dose 50%

LHRH: Luteinizing hormone releasing hormone

LOD: Limit of Detection

LOQ: Limit of Quantitation

LSAB: Labeled Streptavidin Biotin

MDR1: Multidrug resistance 1 gene

MEP: The median-effect principle

MGMT: Methyltransferase

MMR: Mismatch repair

MRP1: Multidrug resistance-associated protein 1

MRT: Mean residence time

MTD: Maximum Tolerated Dose

MX: Mitoxantrone

NCE: New chemical entity

NER: Nucleotide excision repair

NF-κB: Nuclear factor kappa B

NIH: National Institutes of Health

NK: Natural killer cells

PCNA: Proliferating cell nuclear antigen

PDGF: Platelet-derived growth factor

PG: Postprandial glucose

P-gp: P-glycoprotein

PI: postprandial insulin

PIN: Prostatic intra-epithelial neoplasia

PIP_3: Phosphatidylinositol 3,4,5-bisphosphate

pRb: Retinoblastoma protein

PSA: Prostate specific antigen

PTEN: Phosphatase and tensin homolog

QD: Once daily

R.E.: Relative error

RPMI: Roswell Park Memorial Institute

RSD: Relative standard deviation

R.T.:	Room temperature
S.D.:	Standard deviation
S/N:	Signal-to-Noise ratio
S.E.M:	Standard Error of the Mean
S.I.R.:	Single ion recording
$t_{1/}2$:	Half-life
T_{max}:	Time to peak concentration
TGFα:	Tumor growth factor α
TGFβ:	Transforming growth factor β
TMA:	Tissue Micro-Array
TNF-α:	Tumour necrosis factor alpha (TNF-α)
TNM:	Tumor, node, metastasis
TUNEL:	Terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick-end labeling staining
U/L:	Units per litre
U.V.:	Ultraviolet
VEGF:	Vascular endothelial growth factor

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DEDICATION

Ginaga wa Gabede nu Gimweji

CO-AUTHORSHIP STATEMENT

1. FIRST MANUSCRIPT CHAPTER

The research was designed by Alain Musende and reviewed by Drs. Emma Guns and Marcel Bally. Alain Musende carried out: the solubility and stability studies, oral dosing of the mice, animal blood and tissue collection, scanning of the images of tissue microarray cores, AST and ALT analysis, creatinine analysis, the preparation of all solutions, and all data processing and analyses. Andy Eberding performed the intra-venous injection and along with Catherine Wood, helped with the dosing and tissue collection during pharmacokinetic (PK) studies. PK analysis requires team work for timely sacrifice and blood and tissue collection. Hans Adomat contributed in the optimum and efficient operation of the LC/MS/MS instrument. Ladan Fazli and Antonio Hurtado-Coll are the two pathologists, blinded to the study, who performed tissue scoring and analysis for the apoptotic index and Ki-67 level determination.

2. SECOND MANUSCRIPT CHAPTER

Same as in the first manuscript chapter

3. THIRD MANUSCRIPT CHAPTER

The research was designed by Alain Musende and reviewed by Drs. Emma Guns and Marcel Bally. Alain Musende carried out: all *in vitro* work, animal oral dosing and tumor collection, scanning of the images of tissue microarray cores, the preparation of all solutions, and all data processing and analyses. Andy Eberding performed the intra-venous injection, Ladan Fazli and Antonio Hurtado-Coll are the two pathologists, blinded to the study, who performed tissue scoring and analysis for the apoptotic index and Ki-67 level determination.

1. INTRODUCTION

1.1 Thesis overview and objectives

Prostate cancer is the fifth most common neoplasm worldwide, and the second most prevalent cancer among men (Parkin *et al*, 2005). In North America, prostate cancer represents 33% of all new cancer cases making it the most common cancer in men with an incidence of one in six American men who are expected to develop prostate cancer sometime during their lifetime (Delongchamps *et al*, 2006; Edwards *et al*, 2002; Jemal *et al*, 2004; Routh & Leibovich, 2005; Schwartz, 2005). In Canada, prostate cancer constitutes the third leading cause of cancer related deaths, with an estimated 24,700 newly diagnosed cases in 2008 (NCI, 2008). Despite continuous research and multi-million dollars spent every year on research, there are really no curative interventions which are effective when used to treat prostate cancer patients that have relapsed and/or have metastatic disease.

In recent years, however, new drugs and drug combinations and particularly those involving taxanes have contributed to improvements in treatment outcomes in prostate cancer (De Wit, 2008; Oh, 2003). Significantly, accrued clinical evidence suggests a correlation between the number of drugs used as part of a combination regimen and the percent of complete responses or cure rate (Frei & Antman, 1997). Combination therapy offers significant benefits when compared to single-agent therapy by providing: (i) maximal cell kill within the range of tolerable toxicity; (ii) diverse interactions between the drugs procuring pleiotropic effects as is optimal for treating a heterogeneous tumour; and (iii) the potential to prevent or slow the subsequent development of cellular drug resistance. It is also anticipated that combinations of non-toxic effective phytochemicals with established chemotherapy agents could offer the potential of enhanced drug efficacy without adding to the toxicity of the conventional drug. This is also of interest in the

context of complementary medicines, agents that are being used by chemotherapy patients with the belief that they may augment treatment responses achieved by conventional therapy (Richardson *et al*, 2000).

We have assessed the *in vitro* and *in vivo* therapeutic potential of ginsenoside Rh2, a 20(S)-protopanaxadiol type and its aglycone aPPD, alone and in combination with docetaxel. Rh2 and aPPD are *in vivo* metabolic products of ginseng when taken orally. Rh2 has been reported to inhibit the growth of and induce anti-metastatic activity in B16 melanoma and ovarian cancer cells (Jia *et al*, 2004; Nakata *et al*, 1998; Wakabayashi *et al*, 1997). It was also found to have a G1 phase-specific cell cycle arrest with associated suppression of cdk2 and cyclin E-dependent histone kinase activities apparently as a consequence of upregulation of p21 which binds and inactivates cdk2 in MCF-7 human breast cancer cells (Oh *et al*, 1999). aPPD has been reported to inhibit the growth of THP-1 leukemia cells, inducing DNA fragmentation and apoptosis resulting in build up of sub-G1 cells (Popovich & Kitts, 2002). In human breast carcinoma MCF-7/MX cells which overexpress breast cancer resistance protein (BCRP), Rh2 and aPPD significantly enhanced the cytotoxicity of mitoxantrone; an effect that was attributed to inhibition of mitoxantrone efflux and enhanced mitoxantrone uptake. In addition, aPPD was shown to significantly inhibit BCRP-associated vanadate sensitive ATPase activity (Jin *et al*, 2006). Recently, Wang et al. investigated possible mechanisms responsible for the antiproliferative, pro-apoptotic, and cell cycle arrest effects of Rh2 and aPPD against LNCaP androgen-sensitive and PC-3 androgen-insensitive prostate cancer cells. They showed that both Rh2 and aPPD increased the expression of the cdk inhibitor p21 and tumor suppressor protein p53 while decreasing the levels of cdks 2, 4, and 6. The expression of the p53 inhibitor MDM2 was also decreased, along with levels of transcription factor E2F1 (Wang *et al*, 2008c).

Docetaxel is a semi-synthetic taxane drug marketed as Taxotere®, which is prepared from a chemical precursor compound isolated from the needles of the European yew tree, *Taxus baccata*. It acts as a potent inhibitor of cell replication by blocking cells in the G2/M interphase of the cell cycle following stabilization of the microtubule skeleton. Docetaxel inhibits microtubule depolymerization 2-fold greater than its analogue, paclitaxel (Lavelle, 1995). It also exhibits 1.3 to 12-fold greater cytotoxicity, compared to paclitaxel, in several murine and human tumour cell lines. It has also been reported to be retained intra-cellularly for a greater duration (Herbst & Khuri, 2003). In recent years, docetaxel has been recognized as one of the most effective drugs used to treat advanced prostate cancer (Petrylak, 2000).

Four established prostate cancer cell lines are commonly used to investigate the mechanisms involved in the progression of prostate cancer: PC-3, LNCaP, C4-2 and DU145. PC-3 and DU145 cell lines were established from human cancer cell metastasis to the bone and brain, respectively, whereas LNCaP was isolated from a human lymph node metastatic lesion (Krueckl *et al*, 2004; Van Bokhoven *et al*, 2003). The C4-2 cell line was derived upon androgen starvation of LNCaP cells. PC-3, C4-2 and DU145 are androgen insensitive cell lines while LNCaP is androgen sensitive. Since C4-2 is LNCaP-derived, both of these cell lines express the functional tumour suppressor protein p53 as well as functional androgen receptor, albeit mutated from wild type, whereas PC-3 and DU145 are p53 deficient (Ko *et al*, 1996). It has been shown that p53 expression or lack thereof influences apoptosis (Wang *et al*, 2008c).

We have previously shown that Rh2 and paclitaxel behave synergistically in cultured LNCaP cells (Xie *et al*, 2006). In the same study we also reported that oral treatment of LNCaP tumors with Rh2 produced a significant decrease in tumor growth which was accompanied by a

significant decrease in serum prostate specific antigen (PSA) when combined with intravenously administered paclitaxel (Xie *et al*, 2006).

Combination of Rh2 or aPPD with docetaxel follows current dogma which implies that optimal drug combination effects are most likely to be achieved when combining drugs which have different mechanisms of action and different toxicity profiles. The research presented here explores the pre-clinical efficacy of Rh2 and aPPD in the androgen insensitive PC-3 human xenograft model for prostate cancer. Initially, *in vitro* combinations of Rh2 and aPPD with docetaxel, at their IC_{50} ratios, were assessed against the four prostate cancer cell lines described above (PC-3, LNCaP, C4-2, DU145). The combination and drug reduction indices (as indicators of synergism, antagonism, or additivity) were determined. Prior to initiating *in vivo* studies, the solubility and stability of Rh2 and aPPD were determined in ethanol. The ethanol solubilized drugs were formulated for oral gavage with propylene glycol and water at their maximal achievable concentrations. A high performance liquid chromatography tandem mass spectrometry (LC/MS) method was then developed and partially validated for quantitation of each compound in plasma and tissues. This assay was used to determine the pharmacokinetics and biodistribution of Rh2 and aPPD following oral administration to PC-3 xenograft bearing nude mice. The doses used for these pharmacokinetic studies were determined to be well tolerated and effectively inhibited the growth of established PC-3 tumors over a 28 day period. Antitumor activity was also assessed by measuring apoptotic index alongside levels of the proliferation marker Ki-67 in excised tumour specimens. Serum creatinine levels, as a measure of renal toxicity, and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as measures of liver toxicity, were also quantitated following treatment with Rh2. A dose escalation study was later carried out for docetaxel (i.v.) in PC-3 xenograft bearing mice in order to determine an appropriate dose for use in combination studies

with Rh2 and aPPD. The efficacy of Rh2 or aPPD in combination with docetaxel was subsequently assessed in the PC-3 mouse xenograft model.

The first part of this thesis provides pertinent and valuable background information to ensure full comprehension of the work conducted. The background information is organized in four main sections. The first section aims to provide a clear understanding of cancer: its definition and cellular origin, the important genes implicated in genetic changes such as oncogenes, tumour suppressor genes and repair genes, the epigenetic aspect of cancer, critical cellular properties for cancer growth, and general concepts related to cancer therapy. The second section expands the understanding of cancer with a focus on prostate cancer: its epidemiology and risk factors, and its management as influenced by the status of the cancer with respect to androgen dependence or independence. In the third section, the discussion centers on ginseng and ginsenosides: the importance of ginseng and related products in complementary and alternative medicine in North America and a review of pharmacological activities of ginseng, Rh2 and aPPD. The final section discusses the development of drug combination: a review of methods for assessing therapeutic drug-drug interactions, the modulation of drug metabolism and transport processes involved in drug-drug interactions and their toxicological implications, and overall benefits of drug combination in cancer treatment.

1.1.1 Thesis hypothesis

Components within selected natural products can be used as therapeutic agents in combination with existing standard-of-care anticancer medicines to achieve significant improvements in treatment outcomes. More specifically, ginsenoside Rh2 and its aglycone aPPD

can be used in combination with docetaxel to produce enhanced treatment effects in models of prostate cancer.

1.1.2 Objectives

Specific aim 1:

To characterize the effects of Rh2, aPPD, and docetaxel, alone and in combination, on the proliferation of four well characterized human prostate cancer cell lines (PC-3, LNCaP, C4-2, DU145).

Specific aim 2:

To determine the solubility and stability of Rh2 and aPPD in ethanol and formulate them for oral gavage.

Specific aim 3:

To determine the pharmacokinetics and biodistribution of Rh2 and aPPD following oral administration to PC-3 xenograft bearing nude mice.

Specific aim 4:

To assess the toxicity and efficacy of Rh2, aPPD and docetaxel as single agents in the PC-3 mouse xenograft model.

Specific aim 5:

To assess the efficacy of Rh2 or aPPD in combination with docetaxel in the PC-3 mouse xenograft model.

1.2 Cancer: the problem

1.2.1 Multi-cellular organisms

Cells in a multicellular organism live, grow, proliferate and behave in a manner mostly conducive to the overall homeostasis of the tissue/organ and the organism itself, by extension. To achieve this purpose, cells in this environment, unlike unicellular organisms such as bacteria, behave and communicate according to complex regulatory and signaling processes, in order to coordinate their individual behavior. In addition, strict control mechanisms are in place to prevent homeostasis disruption and ensure its infallible continuation. It has been suggested that considering the extremely large number of cells involved, approximately 10^{14} cells in the human body, minor homeostasis disruption due to mutations would be of common occurrence and some, but not all lead to the development of cancer (Alberts *et al*, 2002).

1.2.2 Definition of cancer

Neoplasm, the abnormal new growth of tissue, is categorized as being benign or malignant. It has been observed that benign neoplasms progress into malignant neoplasms in certain types of cancers, such as those of the colon and thyroid, while they remain benign in prostate and breast growths (King & Robins, 2006). Pathological differences between benign and malignant neoplasms are presented in Table 1.1. Cancer refers to malignant neoplasm and is described according to the cell of origin and the tissues in which they occur. Although most common cancers arise in the epithelium (carcinoma), several other types of cells are also affected (King & Robins, 2006). Cancer terminology according to cell type is presented in Table 1.2.

For the aberration in one single cell to give rise to a tumour, it has to be heritable. Aberrations in a cell can be initiated by heritable genetic changes (mutation, alteration in the cell's DNA sequence), like in the vast majority of cancers, or by heritable epigenetic changes (change in the pattern of gene expression without a change in the DNA sequence). Three types of agents are known mutagens: chemical carcinogens (cause simple local changes in the nucleotide sequence), ionizing radiations such as x-ray (cause chromosome breaks and translocations), and viruses (introduce foreign DNA into the cell) (Alberts *et al*, 2002; Peto, 2001).

Most cancers appear when mutations occur in cells which then proliferate while losing their ability to undergo normal programmed cell death or apoptosis.

Table 1.1: Comparison between benign and malignant growths (King & Robins, 2006)

Feature	**Benign**	**Malignant**
Edges	Encapsulated	Irregular
Metastasis	No	Yes
Invasion	No	Yes
Comparison with normal	Good	Variable, often none
Growth rate	Low	High
Nuclei	Normal	Variable, irregular
Life-threatening	Unusual	Usual

Since there is an accumulation of errors and mutations in pre-cancerous cells, and the proliferation of mutated cells requires months or years to reach "cancer" status, many types of cancers are

diagnosed at advanced age (Aapro & Johnson, 2005; Renan, 1993). Mutations at a molecular level are generally of limited impact unless they provide distinct advantages to a single cell over its neighbours, in terms of clonal selection favouring growth, division, and/or proliferation. This process, referred to as carcinogenesis, the process by which cancer is generated, is a multi-step process occurring when mutations and errors accumulate in the regulatory and control pathways within a single cell, ultimately providing the cell involved with additional survival advantages (Renan, 1993).

Table 1.2: Cancer terminology according to cell types (King & Robins, 2006)

Cell type	**General Terminology**	**Specific Terminology**	
Epithelium	Carcinoma	Glandular (i.e. prostate)	Adenocarcinoma
		Squamous (i.e. cervix)	Carcinoma
Mesenchyme	Sarcoma	Smooth muscle Connective tissues	Leiomyosarcoma
		Bone	Osteosarcoma
		Fat cells	Liposarcoma
Nervous system	N/A	Eye	Retinoblastoma
White blood cells	Leukemia.	Lymphocytes	Lymphocytic leukemia

1.2.3 Model of genetic changes in cancer development

Fearon and Vogelstein (1990) proposed a model illustrating precursor genetic alterations required for the progression to carcinoma. This model, referred to as the Vogelgram (Figure 1.1), has been adopted as broadly applicable to epithelial cell derived carcinoma and highlights the role of multiple genetic defects in this process. Vogelstein later identified three groups of genes whose alterations can cause cancer: oncogenes, tumour suppressor genes, and repair genes (Vogelstein *et al*, 2000). These genes and the proteins for which they code describe the molecular functioning of carcinogenesis.

1.2.3.1 Oncogenes

Oncogenes are overactive forms of proto-oncogenes whose product can act in a dominant fashion to help make a cell cancerous. Oncogene activity is increased as a result of genetic changes in either their coding region or regulatory sequences (Murray *et al*, 1981; Pulciani *et al*, 1982). Cells with mutant oncogenes continue to grow (or are unable to to die) even when they are receiving no growth signals (Vogelstein *et al*, 2000). The Ras oncogene which is activated in pancreatic and colon cancers, and Bcl-2 which is activated in prostate and lymphoid cancers, constitute two examples of oncogenes (El-Awady *et al*, 2008; McMurray *et al*, 2008; Milyavsky *et al*, 2005; Wang *et al*, 2008a). For instance, the Ras oncogene bases coding for the sequence of amino acids of its corresponding protein are often mutated in colon cancer. The middle base in the –GGC- codon is mutated to an adenine, resulting in aspartate (GAC) rather than glycine being incorporated into the protein (Yuan *et al*, 2001).

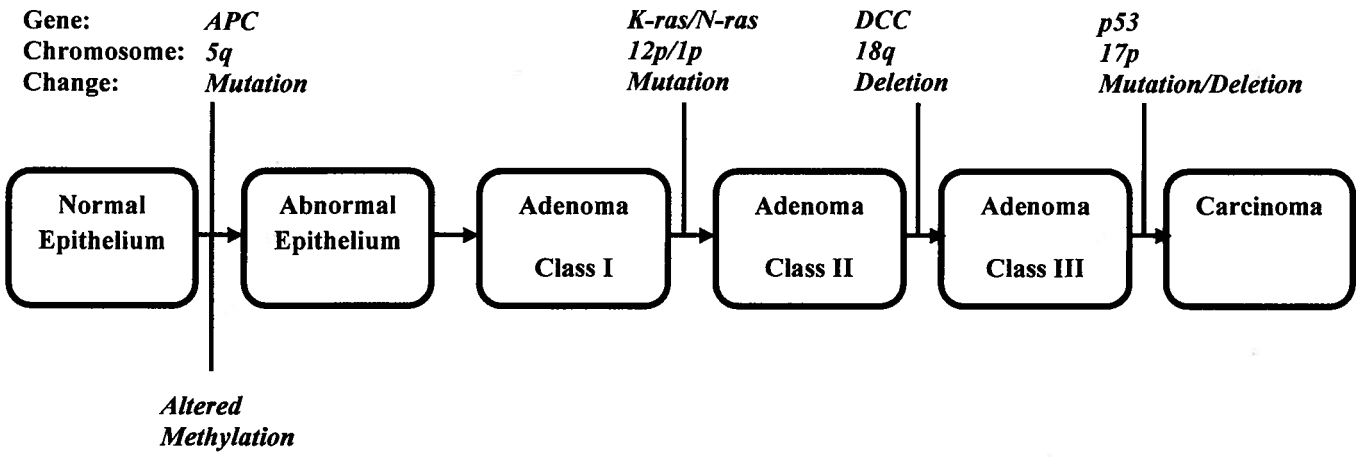

Figure 1.1: "Vogelgram" of molecular genetic alterations during the adenoma-adenocarcinoma sequence based on studies by Vogelstein and colleagues of mucosa, adenomas, and carcinomas of patients with large bowel neoplasia. Mutation of the *APC* gene *(Adenomatous Polyposis Coli)* on the long arm of chromosome 5 leads to abnormal epithelial proliferation and differentiation in patients with adenomatous polyposis syndrome. An increase in the prevalence of *ras* gene mutations, especially in codon 12 of the Kirsten *ras* gene on the short arm of chromosome 12, is evident in intermediate adenomas. Deletion of the *DCC* gene *(Deleted in Colorectal Carcinoma)* on the long arm of chromosome 18 occurs more frequently in late adenomas. Deletion and mutation of the *p53* gene on the short arm of chromosome 17 is associated with the development of carcinomas in adenomas. All these molecular genetic alterations rarely occur together in an individual tumor, and alterations in other genes also have been identified. In addition, this preferential sequence of the alterations is not followed in many tumours, as evidenced by analysis of paired specimens of adenomas and the carcinomas that arose in them. Alterations in the DNA methylation system are evident in the grossly normal colorectal mucosa of patients with large bowel neoplasia and may represent key early events (Adopted by (Hamilton, 1992)).

1.2.3.2 Tumour suppressor genes

Tumour suppressor genes are subject to a loss-of-function mutation, unlike oncogenes which are subject to a gain-of-function mutation. p53, Rb, PTEN, $p16^{INK4a}$, and APC constitute examples of tumour suppressor genes (Ma *et al*, 2005; Mercier *et al*, 2008; Qian *et al*, 2008; Vogelstein *et al*, 2000; Xing *et al*, 1999). Tumour suppressor genes regulate cell numbers, either by inhibiting progress through the cell cycle and thereby preventing cell division, or by promoting programmed cell death (also called apoptosis). They accomplish these functions by complexing with other effector proteins, such as growth promoters, and blocking their action (Kouzarides, 1995; Taya, 1997). Tumour suppressor gene inactivation is achieved by preventing its binding to effector genes, a process that can be achieved in several ways including phosphorylation and mutation of the suppressor gene. Phosphorylation of Rb at serine/threonine leading to inhibition of its binding to a transcription factor is an example (Chao *et al*, 1992; Huang *et al*, 1988). p53, considered the guardian of the genome, is arguably the most frequently altered gene in human cancers. It achieves protection of the genome by coordinating the blockade of cell proliferation, stimulation of DNA repair, and promotion of apoptosis (Lane, 1994; Martinez *et al*, 1991; Yonish-Rouach *et al*, 1991). p53 regulates the cell fate in response to genotoxic (DNA alterations induced by irradiation, UV, carcinogens, cytotoxic drugs) or non genotoxic (hypoxia, nucleotide depletion, oncogene activation, microtubule disruption, loss of normal cell contacts) stresses (Gorgoulis *et al*, 2005; Graeber *et al*, 1994; Vousden & Lu, 2002).

DNA damage has been shown to lead to rapid induction of p53, which induces either apoptosis or activation of the transcription of p21 (Bae *et al*, 1995). As a cdk inhibitor, p21 blocks cell cycle progression through inhibition of both Cdk/cyclin complexes and DNA replication by binding to proliferating cell nuclear antigen (PCNA) (Li *et al*, 1994; Zhang *et al*, 1993). It is

assumed that the resulting cell cycle arrest would allow for damaged DNA to be repaired before it is replicated. Unrepaired DNA damage normally induces apoptosis in mammalian cells, thereby eliminating cells carrying potentially deleterious mutations (Devlin *et al*, 2008; Levine *et al*, 1994). Lack of p53 prevents this damage-induced cell cycle arrest or apoptosis to occur, with two consequences: (i) increased mutation frequencies and a general instability of the cell genome and (ii) reduced apoptosis. Genetic instability is common in cancer cells and contributes to further mutations activating new oncogenes and deactivating new tumour suppressor genes during tumor progression (Sarasin, 2003). Cancer cells fail to undergo apoptosis as a response to DNA damage induced by radiation, DNA damaging chemicals or oxygen deprivation thus contributing to resistance to chemotherapy (Clarke *et al*, 1993; Lowe *et al*, 1993; Weinstein *et al*, 1997).

The tumour-suppressor gene PTEN, the phosphatase and tensin homologue gene frequently deleted from chromosome 10 is a central regulator of brain, breast, and human prostate cancer (Li *et al*, 1997a; Myers *et al*, 1997; Steck *et al*, 1997). The protein encoded by this gene is a lipid phosphatase that dephosphorylates the 3 position of phosphatidylinositides, such as phosphatidylinositol 3,4,5-bisphosphate (PIP_3). PIP_3 dephosphorylation inhibits the activities of PI3-kinase and Akt (Bellacosa *et al*, 1991; Chang *et al*, 1997). Inactivation or loss of the PTEN tumour suppressor protein can contribute to tumor development as a result of increased levels of PIP_3, activation of Akt, and inhibition of programmed cell death. Li et al. detected homozygous deletions, frameshift, or nonsense mutations in PTEN in 63% of glioblastoma cell lines, 100% of prostate cancer cell lines, and 10% of breast cancer cell lines (Li *et al*, 1997a). PTEN is lost in 23% of high grade PIN (prostatic intraepithelial neoplasia), and 68% of prostate adenocarcinomas (Yoshimoto *et al*, 2006). PTEN deficiency confers protection from cell death and resistance to chemotherapy of prostate cancer cells (Wang *et al*, 2004).

Since most cancers arise in somatic cells, which are dipoid (they carry two copies of each autosomal genes (allele)), it has been shown that a mutation in one allele only, can result in dominant gain-of-function, and is characteristic of oncogenes (Capon *et al*, 1983; Sukumar *et al*, 1983). Although tumour-suppressor genes such as p53 suppressors also require only a single allelic mutation to become altered, other suppressor genes such as Rb must be mutated in both alleles to lose their normal function (Boynton *et al*, 1991; Kohler *et al*, 1993). p53 is a transcription factor that requires two of its molecules to bind and form a homodimer in order to be activated. A single allelic mutation in one of the p53 molecules leads to the formation of an inactive heterodimer (Tarunina & Jenkins, 1993).

1.2.3.3 Repair genes

Repair genes have a much more fundamental role with respect to cancer development than oncogenes and tumor suppressor genes: they monitor the rate of mutations of all genes, including the oncogenes and tumor suppressor genes discussed above. When repair genes are themselves mutated, acquisition of mutations by oncogenes and tumour suppressor genes is accelerated, leading to neoplasm or the formation of a tumor. Three groups of DNA repair genes are associated with distinct repair mechanisms: mismatch repair (MMR), base excision repair (BER), and nucleotide excision repair (NER). Inactivation of repair genes such as nucleotide-excision repair genes and mismatch-repair genes often leads to skin and colon tumours, respectively. (i) MMR is responsible for removal of base mismatches caused by spontaneous and induced base deamination, oxidation, methylation and replication errors. MMR targets mostly base mismatches such as G-T, G-G, A-C, and C-C (Fang & Modrich, 1993; Modrich & Lahue, 1996; Umar & Kunkel, 1996). (ii) BER genes remove DNA-damaged bases, which can be recognised by specific enzymes, the DNA glycosylases.

The main lesions subjected to BER are oxidised DNA bases, caused by inflammatory responses, or exposure to exogenous agents such as ionising radiation and long-wave UV light. Another main source of lesions repaired by BER is DNA alkylation induced by endogenous alkylating species and exogenous carcinogens such as nitrosamines and various anticancer drugs (Dianov *et al*, 1992; Matsumoto & Kim, 1995; Scharer & Jiricny, 2001). (iii) NER are responsible for the repair of Bulky DNA adducts such as UV-light-induced photolesions, intrastrand cross-links, large chemical adducts generated from exposure to aflatoxine, benzo(*a*)pyrene and other genotoxic agents (Friedberg, 2001; Hanawalt, 2001).

1.2.3.4 Viral carcinogenesis

Viruses are also known to induce alteration of cell function and cause cancer. They can be categorized in two groups: RNA and DNA viruses. RNA viruses use reverse transcription to infect competent cells and are appropriately classified as retroviruses. They convert their RNA into DNA which they incorporate into the host genome. Briefly, shortly after infection, the viral RNA genome is transcribed by a virion-associated enzyme, the reverse transcriptase, into a double-stranded DNA copy which is then integrated into the chromosomal DNA of the cell with the help of the viral integrase enzyme. The integrated copy, termed provirus, is almost never lost from the chromosome, leading to the conclusion that retroviral infection of a cell is permanent (Baltimore, 1970; Stehelin *et al*, 1976; Temin & Mizutani, 1970). Examples of RNA viruses include the human immunodeficiency virus (HIV), and the human T-cell lymphotropic virus (HTLV), which causes leukaemia (Gurgo & Gallo, 1987). DNA viruses use their own protein products to directly bind and inactivate host proteins, such as the tumour suppressor proteins p53 and Rb, which can lead to altered cell proliferation. In this case, virus-encoded non-structural proteins, such as SV40 large T-

antigen, stimulate resting cells to enter the S phase and provide the enzymes and environment necessary for viral DNA replication (Pipas, 1992). The human papilloma virus (HPV) which causes cervical cancer generates its E7 oncoprotein which binds and subsequently inactivates the Rb suppressor protein (pRb) (Barbosa *et al*, 1990). Other DNA viruses that are important in human cancers include: hepatitis B virus (HBV) in liver cancer, the Epstein-Barr virus (EBV) in Burkitt's lymphoma and nasopharyngeal cancers, and the Kaposi sarcoma virus (KSV) in Kaposi's sarcoma (Cianciara, 1975; Papaevangelou *et al*, 1979; Walen & Arnstein, 1986). Approximately 30 HPVs cause infection of the genital tract. Of this group, HPV 16, 18, 45, and 56 greatly increase the risk of developing cervical cancer and generate proteins E6 and E7 (King & Robins, 2006). Development of cervical cancer requires the presence of the viral E6 and E7 sequences (Ledwaba *et al*, 2004; Munger *et al*, 2004). There is normally a relatively long time between infection and the development of cervical cancer. It is assumed that cofactors such as genotoxic carcinogens in tobacco smoke and progestins in oral contraceptives favour progress of the disease. Progestins, in combination with receptor proteins bind and activate HPV promoter sequences (Bosch *et al*, 1992; Hildesheim *et al*, 2001; Kjaer *et al*, 1996). Chronic HBV infection causes cirrhosis of the liver, a chronic inflammation vulnerable to successive action by cofactors such as alcohol and aflatoxins (Donato *et al*, 2006; Ikeda *et al*, 1998). Liver cancer progression follows the following steps: inflammation, increased proliferative activity (dysplasia), dysplastic nodules, carcinoma and metastasis (Hytiroglou *et al*, 2007). The development of liver cancer takes up to 20 years, which implies that cofactors are necessary for the progression. Enhanced cell proliferation and reduced cell death results from the upregulation of the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) in adjacent endothelial and inflammatory cells which activates the transcription factor nuclear factor kappa B (NF-κB) (Barnhart & Peter, 2003).

1.2.4 Invasion and metastasis

The tumour is considered malignant once it has acquired the capability of invading surrounding tissues. It is important to keep in mind that since cancer formation is a multi-step process, it is traditionally believed that cells become malignant only through progressive acquisition of new mutations combined with clonal selection, favouring clones with dominant attributes such as faster growth rate (Fidler & Talmadge, 1986; Foulds, 1954; Hart & Easty, 1991). Cancer cells later escape from the primary tumour site, infiltrate lymphatic or blood vessels, and are transported to distant sites where they establish secondary tumours. Progression to this later stage, called metastasis, is illustrated in Figure 1.2 and involves the following steps: migration, intravasation, circulation, extravasation, and growth at the secondary site.

Metastasis remains the primary cause of death among cancer patients (Molloy & van 't Veer, 2008). Attempts at elucidating the concept of metastasis, such as the seminal "seed and soil" hypothesis, were published as early as 1889 by the English surgeon Stephen Paget. He concluded that: (i) certain cells from a primary tumour act as seeds and have specific affinity for the milieu of certain organs ("soil"). (ii) metastases were formed only when the seed and soil were compatible (Paget, 1889). It has demonstrated that sites of metastasis are determined not solely by the characteristics of the neoplastic cells but also by the microenvironment of the host tissue (Hart & Fidler, 1980).

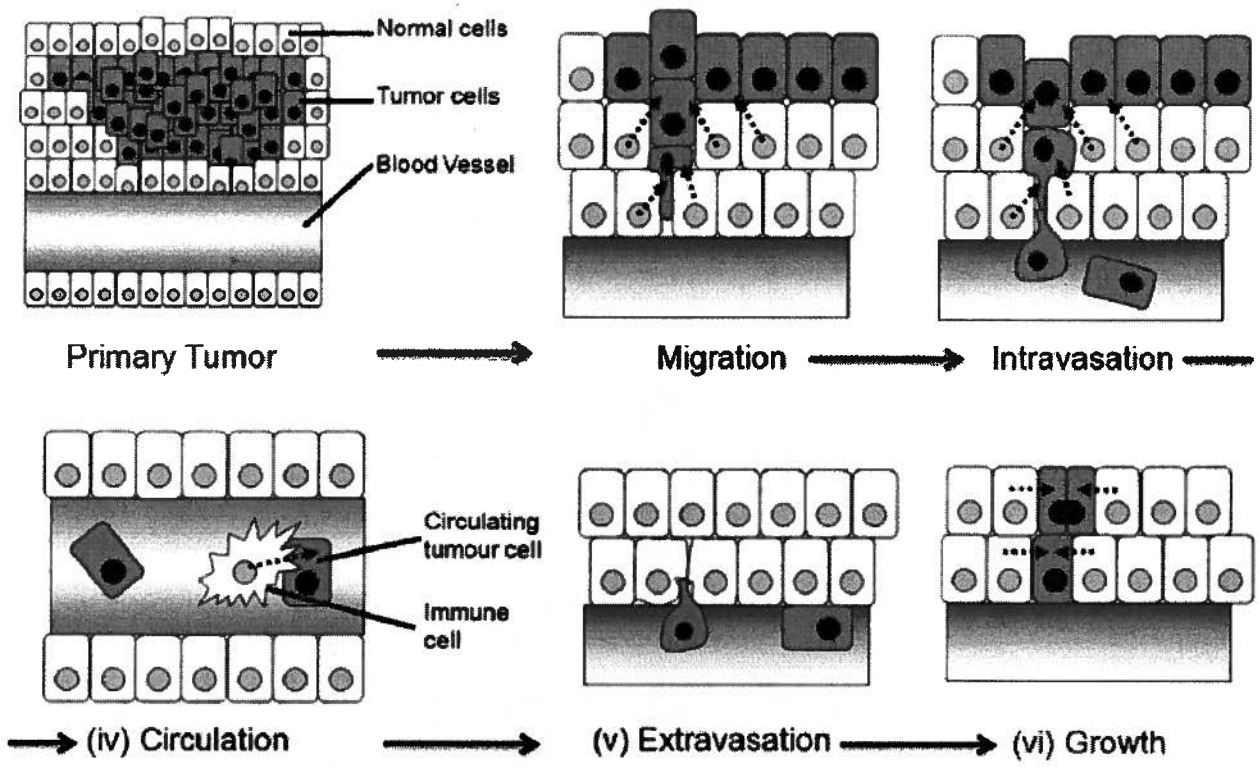

Figure 1.2: Summary of the metastatic process. From a primary tumor, a 'metastasis-permissive' microenvironment is formed in which surrounding healthy cells are induced to secrete signaling molecules which promote growth and migration of the cancer cells (denoted by dashed arrows). The loss of local constraints may promote epithelial–mesenchymal transition (EMT) and allows tumor cells to access the circulatory or lymphatic system, where they must negotiate both host defenses and physical stresses. Eventually, tumor cells interact with the surface antigens of cells making up the vessel walls at a remote location which stimulates changes in their morphology allowing extravasation. Once inside the tissue parenchyma of the distant organ, the tumor cell will once again take advantage of paracrine signaling by the surrounding healthy tissue to remodel the area and create a niche suitable for overt growth (Taken from (Molloy & van 't Veer, 2008)).

More recent research suggest that: (i) metastatic capability may rather be an intrinsic feature of tumors which is acquired much earlier in the progression of the disease (Bernards & Weinberg, 2002), and (ii) tumor cells are significantly dependant on normal cells in the immediate microenvironment and the immune system for disease progression (Anderson *et al*, 2006; Chang *et al*, 2007; Koizumi *et al*, 2007; Walser *et al*, 2007)

1.2.5 Epigenetic events in cancer

It is widely accepted that the initiation and progression of human cancer is caused by loss of tumor suppressor function. Inactivation of tumor suppressor genes can result from both genetic mechanisms such as mutations and epigenetic mechanisms such as DNA hypermethylation (Chan *et al*, 2008). In the mammalian genome, methylation takes place only at cytosine bases that are located 5' to guanosine in a CpG dinucleotide (Bird, 2002). DNA methylation is a predominant mode of epigenetic alteration in cancer involving gene silencing via CpG island promoter hypermethylation (Antequera *et al*, 1990; Jones *et al*, 1990). Hypermethylation has now been observed to result in abnormal silencing of a number of tumor suppressors in many human malignancies such as cancers of the breast, prostate, colon, lymphoid, liver, and lung (Esteller *et al*, 2000; Herman *et al*, 1997; Lin *et al*, 2001; Wales *et al*, 1995). Promoter hypermethylation is considered to be at least as common as, and possibly more than, the disruption of classic tumour-suppressor genes in human cancer by mutations (Herman *et al*, 1998; Ohtani-Fujita *et al*, 1997; Prowse *et al*, 1997).

Epigenetic abnormalities can also cooperate with genetic alterations to effect aberrant gene function that result in cancer (Chan *et al*, 2008). In fact, there is a growing awareness that epigenetic gene silencing in cancer can predispose to mutational events during tumour progression. The O^6-methylguanine-DNA methyltransferase (*MGMT*) protein is an example of a DNA-repair gene that is silenced in association with promoter methylation in colon, lung, lymphoid and other tumours (Esteller *et al*, 1999; Esteller *et al*, 2001; Esteller *et al*, 2000). O^6-*MGMT* protein removes carcinogen-induced O^6-methylguanine adducts from DNA. Carcinogen-induced O^6-methylguanine adducts induce a G to A (guanine to adenine) transition mutation if left unrepaired (Nakamura *et al*, 2001; Zuo *et al*, 2004). Tumours with *MGMT* alleles seem to be predisposed to mutations in key

genes such as tumour suppressor protein p53 (Esteller *et al*, 2001) and K-ras (Esteller *et al*, 2000). This promoter methylation seems to precede genetic changes by occurring in pre-malignant polyps that do not yet harbor gene mutations (Esteller *et al*, 2001; Esteller *et al*, 2000).

It is important to recognize that cytosine methylation can influence tumorigenicity by mechanisms other than gene-silencing. These mechanisms take place because 5-methylcytosine is itself mutagenic: it can undergo spontaneous hydrolytic deamination to cause C to T (cytosine to thymine) transition (Coulondre *et al*, 1978). During the development of skin cancer, the presence of the methyl group in the CpG dinucleotides in the coding region of the p53 gene strongly increases the rate at which mutations are induced by ultraviolet (UV) light (Pfeifer *et al*, 2000). According to the biochemical mechanism of this enhancement, the methyl group shifts the UV absorption spectrum for cytosine to a region in the spectrum that is prevalent in sunlight (Jones & Baylin, 2002). Methylated CpG dinucleotides are also the preferred targets of G to T transversion mutations, which are induced in mammalian cells by the tobacco carcinogen benzo(a)pyrene diol epoxide (Yoon *et al*, 2001). Thus, the methylation that occurs in the transcribed region of the p53 gene increases its susceptibility to spontaneous deamination, UV-induced mutation and hydrocarbon carcinogenesis. In order to understand how promoter hypermethylation participates in the loss of gene transcription, it has been observed that in experimental systems, methylation of promoter regions does not lead to silenced transcription until chromatin proteins are recruited to the region, which then mediate gene silencing (Kass *et al*, 1997). Methylation seems to only initiate the process that results in a loss of transcription. Whatever the sequence of events at a given promoter, it seems that promoter methylation must be integral to the loss of gene function because drugs that induce DNA de-methylation such as 5-AZA-2'-DEOXYCYTIDINE, can partially reactivate

silenced genes in cancer cells to restore their function (Baylin & Herman, 2000; Jones & Laird, 1999).

1.2.6 Cardinal properties for cancerous growth

In order for a benign tumour to become malignant and metastasize, the cells need to acquire a distinct set of characteristics, abherrant from normal cells. Hanahan et al. have developed a set of cell physiology traits that enable malignancy and progression to metastasis (Hanahan & Weinberg, 2000): self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Figure 1.3).

1.2.6.1 Cancer cells develop self-sufficiency in growth signals

Normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules (Kang *et al*, 2008; Nagpal *et al*, 2008; Prevost *et al*, 2002).

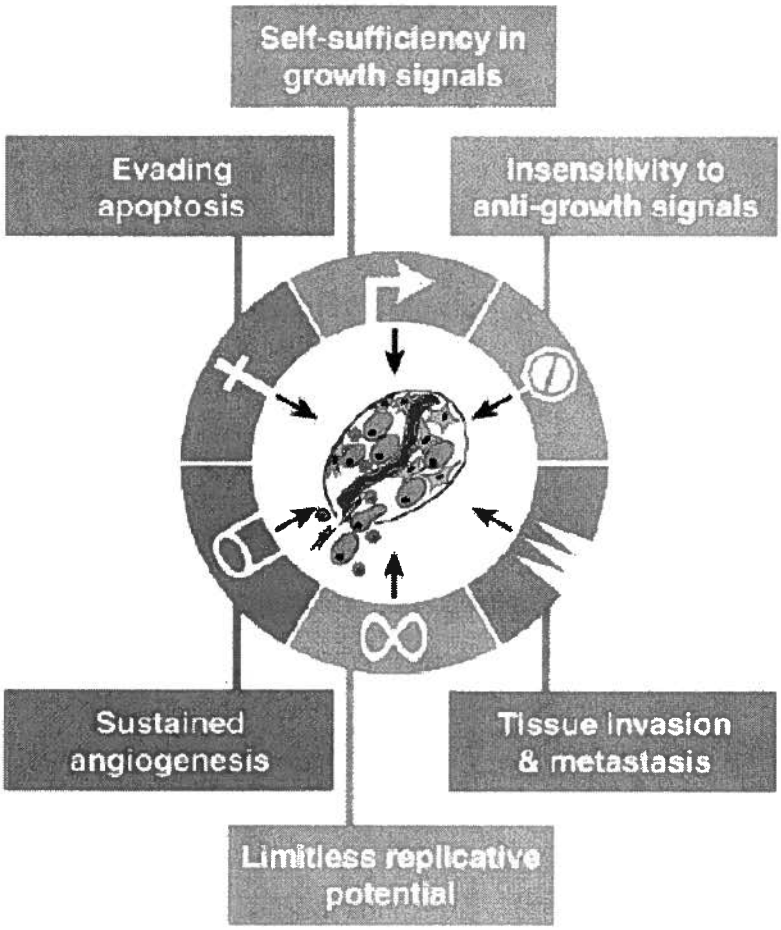

Figure 1.3: Cardinal properties for cancer growth. self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Adapted from (Hanahan & Weinberg, 2000))

No type of normal cell can proliferate in the absence of such stimulatory signals. Many oncogenes act by mimicking normal growth signal in some capacity (Datta *et al*, 2007; Preto *et al*, 2008). Exogenous generation and transduction of most growth factors is heterotypic, implying that the growth factor is produced by one type of cells for the proliferative stimulus of a different type of cells. Cancer cells develop a considerable level of proliferative autonomy, expressed by their

minimal requirement for extracellular growth stimulatory signals. Three common molecular strategies for achieving this autonomy are evidenced involving:

(i) Alteration of extracellular growth signals: many cancer cells acquire the ability to synthesize growth factors (GF) to which they are responsive, creating a positive feedback signaling loop often termed autocrine stimulation (Alexander & Currie, 1984; Ikeda *et al*, 1982). Cancer cells thus obviate dependence on GFs from other cells within the tissue by manufacturing their own GFs. Two illustrative examples of this autonomy are the production of PDGF (platelet-derived growth factor) and TGFα (tumor growth factor α) by glioblastomas and sarcomas, respectively (Aasland *et al*, 1990; Pantazis *et al*, 1985).

(ii) Alteration of transcellular transducers of those signals: two types of alterations of cell surface receptors have been identified, receptor overexpression and switch. Receptor overexpression may enable the cell to become hyperresponsive to ambient levels of GF that normally would not trigger proliferation (Libermann *et al*, 1985; Merlino *et al*, 1984). GF (EGF-R/*erbB*) and HER2/*neu* receptors constitute two illustrative examples. The epidermal GF receptor (EGF-R/*erbB*) is upregulated in stomach, brain, and breast tumors, while the HER2/*neu* receptor is overexpressed in stomach and mammary carcinomas (Slamon *et al*, 1987; Yarden & Ullrich, 1988). Additionally, gross overexpression of GF receptors can elicit ligand-independent signaling (Di Fiore *et al*, 1987). Ligand-independent signaling can also be achieved through structural alteration of receptors; for example, truncated versions of the EGF receptor lacking much of its cytoplasmic domain fire constitutively (Fedi *et al*, 1997). Cancer cells are also known to switch the matrix receptors that they express, such as integrins, in favour of pro-growth signal transmitter types (Lukashev & Werb, 1998).

(iii) Alteration of intracellular circuits that translate those signals into action: this constitutes the most complex mechanism of acquired self-sufficiency in growth signals. Alterations in components of the downstream cytoplasmic circuitry that receives and processes the signals emitted by ligand-activated GF receptors and integrins, enable them to release a flux of mitogenic signals into cells, without ongoing stimulation by their normal upstream regulators. The SOS-Ras-Raf-MAPK cascade plays a central role here. For example, in about 25% of human tumors (Medema & Bos, 1993), Ras proteins are present in structurally altered forms, that enable them to stay in the "on" mode, generating mitogenic signals without prior activation by the upstream Sons of Sevenless (SOS) protein (Bonfini *et al*, 1992).

1.2.6.2 Cancer cells are insensitive to antigrowth signals

As described in Section 5.1, within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. These signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix (ECM) as well as the surface of nearby cells. These growth-inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signaling circuits (Aggarwal *et al*, 1994; Greiner *et al*, 1985). They apply their antiproliferative actions by forcing proliferating cells either temporarily into the quiescent (G_o) state or permanently into post-mitotic stages. For cancer cells to survive and proliferate they must adapt to a stage where the normal anti-proliferative signals are no longer working. Cells monitor their external environment during the G1 phase and, on the basis of sensed signals, decide whether to proliferate (move into S phase), to be quiescent (G_o), or to enter into a post-mitotic state (Denhardt *et al*, 1986; Hartwell & Weinert, 1989; Murray & Kirschner, 1989; O'Farrell *et al*, 1989). At the molecular level, one of the

key cell cycle checkpoint proteins is the retinoblastoma protein (pRb; see section 3.2) and its two relatives, p107 and p130 (Cobrinik *et al*, 1993; Kiess *et al*, 1995). Many and perhaps all antiproliferative signals are funneled through these proteins (Weinberg, 1995). When in a hypophosphorylated state, pRb inhibits cell cycle progression and blocks proliferation by binding E2F transcription factors that control the expression of many genes essential for progression from G1 into S phase (Helin *et al*, 1992; Kiess *et al*, 1995). The activities of pRb are inhibited via phosphorylation by cyclin:CDK complexes, thereby allowing for unimpeded cell proliferation, catalyzed by the transcriptional activity of E2F. Phosphorylation of pRb is inhibited by transforming growth factor β (TGFβ) (Bates *et al*, 1994; Cobrinik *et al*, 1993; Hatakeyama *et al*, 1994).

1.2.6.3 Cancer cells evade apoptosis

As outlined above, in order to progress to malignancy cancer cells need to develop the ability of unobstructed proliferation as well as an ability to avoid programmed cell death through apoptosis. Unlike in necrosis where a dying cell is injured, bursts and spills its content, damaging neighbouring cells; in apoptosis the cell death process is organized and eventually leads to the dying cell being engulfed by macrophages or neighbouring cells (Kerr *et al*, 1972; Saladino & Trump, 1968). Apoptosis is characterized by DNA fragmentation, chromatin condensation, nuclear rupture, cell shrinkage, followed by cell rupture into apoptotic bodies (MacKenzie & Clark, 2008). The molecular mechanism of apoptosis involves two integral components: sensors and effectors. Sensors essentially conduct surveillance of the inner and outer cellular environment, pinpointing situations requiring initiation of apoptosis. Examples of sensors include FAS receptors (binds FAS ligand) and TNF-R1 receptors (binds TNFα) through which death signals are transmitted

(Ashkenazi and Dixit, 1999). Effectors are the executioners of apoptosis, following analysis and detection by sensors. The ultimate effectors of apoptosis include an array of intracellular proteases termed caspases (Thornberry & Lazebnik, 1998). Two "gatekeeper" caspases, 8 and 9, are activated by death receptors such as FAS or by the cytochrome C released from mitochondria, respectively (Imai *et al*, 1999; Li *et al*, 1997b; Muzio *et al*, 1996). These proximal caspases trigger the activation of a dozen or more effector caspases that execute the death program, through selective destruction of subcellular structures and organelles, and of the genome (Fernandes-Alnemri *et al*, 1994). Two other important components of the cellular apoptotic machinery are the Bcl-2 family of proteins (i.e. Bax) which controls cytochrome C release, and the p53 protein which upregulates Bax following DNA damage (Miyashita *et al*, 1994; Selvakumaran *et al*, 1994). The p53 tumour suppressor gene, which also triggers apoptosis under hypoxic conditions, is defective/mutated in more than 50% of human cancers (Harris, 1996). Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. The most commonly occurring loss of a pro-apoptotic regulator involves the *p53* tumor suppressor gene. Functional inactivation of its product, the p53 protein removes a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Harris, 1996). Another mechanism for evading apoptosis involves the PI3 kinase-Akt/PKB pathway, an anti-apoptotic pathway most likely up-regulated in cancer. This pathway is activated by Ras (Downward, 1998) and the loss of the PTEN tumour suppressor gene (Cantley & Neel, 1999).

1.2.6.4 Cancer cells develop limitless replicative potential

Self-sufficiency in growth signals, insensitivity to antigrowth signals, and the ability of evading apoptosis do not suffice in providing cells an ability to replicate endlessly. It is thought

that the ability to acquire limitless replicative abilities beyond a maximum number of cell doublings occurs only after experiencing senescence (lost of the ability to divide) followed by a state of crisis (massive cell death) (Wright *et al*, 1989). The crisis state can be understood at a molecular level by observing the fate of telomeric DNA. Since every cell cycle replication causes telomeric DNA loss at both ends of the chromosomes, chromosomal ends fuse, inducing crisis-like state followed by cell death (Counter *et al*, 1992). It is believed that cancer cells achieve limitless replicative ability through an acquired ability to maintain telemore length. The cancer cell appears to develop two strategies: (i) Addition of hexanucleotide repeats at the end of telomeric DNA via up-regulation of telomerase enzyme (Kruk *et al*, 1996) and (ii) recombination-based inter-chromosomal exchanges of sequence information through activation of a complex mechanism called Alternative lengthening of telomeres (ALT) (Bryan and Cech, 1999; Bryan et al., 1995).

1.2.6.5 Tumors require sustained angiogenesis

Most cells of a tissue are located within a 100 μm perimeter of a capillary blood vessel, mainly to allow for the provision of oxygen and nutrient supply. The process of growing new blood vessels, referred to as angiogenesis, is monitored by a strict regulation of the balance between angiogenesis-promoting and angiogenesis-inhibiting factors (Larcher *et al*, 1996; McLeskey *et al*, 1998; Zhang *et al*, 1995). Angiogenesis-promoting factors include the vascular endothelial growth factor (VEGF) (Phillips *et al*, 1976; Wolf & Harrison, 1973) and the fibroblast growth factor (FGF 1/2) (Gospodarowicz, 1975). These factors bind to transmembrane tyrosine kinase receptors, the VEGF receptor and the FGF receptor (Hanahan & Weinberg, 2000). Thrombospondin-1 is an example of angiogenesis-inhibiting factor and is bound to the CD36 trans-membrane receptor (Murphy-Ullrich & Mosher, 1987; Schuepp *et al*, 1991). It is believed that cancer cells initiate

angiogenesis at the premalignant stage, through disturbance of the regulatory balance between angiogenesis-promoting and angiogenesis-inhibiting factors (Hanahan & Folkman, 1996). As evidenced in several tumours, this is accomplished by either upregulation of VEGF and/or FGF 1/2 or downregulation of thrombospondin-1. Interestingly, it has been demonstrated that decrease in p53 regulation causes substantial downregulation of thrombospondin-1 (Dameron *et al*, 1994) while VEGF is upregulated following the loss of function of the Ras oncogene (Larcher *et al*, 1996).

1.2.6.6 Tissue invasion and metastasis

Metastasis is known to be responsible for 90% of human cancer death (Sporn, 1996). Normal cells are fastened to their environment as a result of the effect of two groups of proteins: the cell-cell adhesion molecules (CAM) and integrins which act as a link to the extracellular matrix (ECM). Cancer cells undergo invasiveness and metastasis through alterations of these two types of proteins. CAMs include the immunoglobulin and calcium-dependent cadherin families (Albelda & Buck, 1990). The most widely observed alteration in cell-to-environment interactions in cancer involves the epithelial transmembrane protein E-cadherin, a homotypic cell-to-cell interaction molecule ubiquitously expressed on epithelial cells. Coupling between adjacent cells by E-cadherin bridges results in the transmission of antigrowth and other signals via cytoplasmic contacts with β-catenin to intracellular signaling circuits that include the Lef/ Tcf transcription factor (Christofori & Semb, 1999). E-cadherin function is apparently lost in a majority of epithelial cancers, by mechanisms that include mutational inactivation of the E-cadherin or β-catenin genes, postranscriptional repression, or proteolysis of the extracellular cadherin domain (Christofori & Semb, 1999). Changes in integrin expression are also evident in invasive and metastatic cells. Invading and metastasizing cancer cells are exposed to varying tissue microenvironments as they migrate, which can present novel matrix components. Accordingly, successful colonization of these

new sites (both local and distant) demands adaptation, which is achieved through shifts in the spectrum of integrin α or β subunits displayed by the migrating cells (Hapke *et al*, 2001). These novel permutations result in different integrin subtypes (of which there are greater than 22) having distinct substrate preferences (Eble & Haier, 2006). Thus, carcinoma cells facilitate invasion by shifting their expression of integrins from those that favor the ECM present in normal epithelium to other integrins (e.g., α3β1 and αVβ3) that preferentially bind the degraded stromal components produced by extracellular proteases (Lukashev & Werb, 1998; Varner & Cheresh, 1996).

1.2.7 Cancer therapy

General cancer treatment considerations include early detection, cellular heterogeneity, and drug resistance. Early detection of very small or premalignant tumors through screening methods is expected to yield the most favourable outcome of cancer treatment (Srinivas *et al*, 2001; Verma & Srivastava, 2003) while the cellular heterogeneity of a tumour contributes to different responsiveness to treatment (Galmarini & Galmarini, 2003; Honeth *et al*, 2008). In addition, various mechanisms enable cancer cells to acquire resistance to a drug notably, overexpression of P-gp or MRP1 efflux transporters, amplification of the gene of the target protein of a drug, the complex yet efficient ability of cells to repair their DNA following damage from drugs, and drug accessibility to tumor cells far removed from blood vessels (Galmarini & Galmarini, 2003; Minchinton & Tannock, 2006; Pajouhesh & Lenz, 2005).

Cytotoxic drugs used in chemotherapy include synthetic agents and natural products. Synthetic agents include (i) Alkylating agents which disrupt DNA synthesis by forming adducts with DNA bases such as guanine. This group includes drugs such as cisplatin and its analog, carboplatin (Stone et al, 1974; Wheeler, 1962). (ii) Antimetabolite drugs which have various

mechanisms of inhibiting nucleic acids. This group includes methotrexate, 5-fluorouracil (5-FU), and cytarabine (Heidelberger et al, 1957). Natural products can be divided in three groups. (i) Topoisomerase inhibitors which inhibit topoisomerases I and II, enzymes responsible for the uncoiling of DNA prior to replication and includes drugs such as topotecan and etoposide. (ii) Microtubule poisons which induce apoptosis via inhibition of chromosome separation, following disruption of the microtubule spindle during mitosis, notably the vinca alkaloids and the taxanes. Vinca alkaloids prevent the polymerization of tubulin and include: vincristin, vinblastine, and vinorelbine (Jordan et al, 1991; White, 1968; Wilson et al, 1999). Taxanes, on the other hand, stabilize the microtubules by preventing their depolymerization (Bhalla, 2003; Schiff & Horwitz, 1980). The two taxanes in clinical use currently are paclitaxel and docetaxel. Microtubule poisons are effective against many types of cancers including prostate, leukemia, lymphoma, ovarian, breast, and testicular (Gidding et al, 1999; Guastalla & Dieras, 2003; Lonnerholm et al, 2008; Oh, 2003). (iii) Anthracyclin antibiotics damage DNA in four ways: they cause partial unwinding of the double strand, generate free radicals, cause single and double strand breaks, and bind to topoisomerase II. They include drugs such as doxorubicin and epirubicin (Minotti et al, 2004).

1.3 Prostate cancer

1.3.1 Epidemiology and risk factors

Prostate cancer is the fifth most common neoplasm worldwide, and the second most prevalent cancer among men. Of all new cancer cases in 2002, 679,000 of them were prostate cancer which resulted in 221,000 deaths (Parkin *et al*, 2005). Prostate cancer is the third leading cause of cancer related deaths in Canada, with an estimated 24,700 newly diagnosed cases in 2008 (NCI, 2008). One in six American males are expected to develop prostate cancer sometime during

their lifetime, contributing to the findings that it is the most common cancer in men in North America, accounting for 33% of all new cancer cases (Delongchamps *et al*, 2006; Edwards *et al*, 2002; Jemal *et al*, 2004; Routh & Leibovich, 2005; Schwartz, 2005). The mortality rate in North America for prostate cancer patients is approximately 9% (Jemal *et al*, 2004).

Established risk factors associated with prostate cancer include: age, ethnic background, diet, and genetics. The risk of developing prostate cancer has been shown to increase with age. In addition, a family history of prostate cancer is also associated with increased risk of prostate cancer, as one first degree relative (father, brother, or son) with the disease increases the risk two-fold, and two first degree relatives further increases the relative risk to 8.8 (Klein, 1995). Men of African descent have a relative risk of 1.8 over men of European descent (Morton, 1994), and men from Asia have the lowest risk, suggesting that ethnic background is also a risk factor. The incidence of prostate cancer is quite low in most Asian countries, including Japan (Parkin *et al*, 2005). However, an increase in incidence is observed in individuals who emigrate from a low-risk country like Japan to a high-risk country like the United States (Parkin *et al*, 2005). Second generation Asian-Americans have increased risk, indicating that lifestyle and environment are important. Diet has been shown to be an important determinant of risk since a higher fat diet has a relative risk of 1.3 (Key, 1995). In addition, dietary components such as lycopene, green tea, vitamin E, and selenium have all been shown to have chemopreventive effects (Reviewed in Gupta, 2006). Considering that these compounds are antioxidants, it is suggested that they protect cells from DNA damage. This is particularly interesting, considering that expression of glutathione-S-transferase (GST), which prevents oxidative stress-induced DNA damage, is commonly lost early in prostate cancer (Brooks *et al*, 1998; Lee *et al*, 1994; Millar *et al*, 1999). Although genetic predisposition of prostate cancer

is not well understood, studies have suggested that it contributes to nearly 50% of prostate cancer cases (Carter *et al*, 1992; Lichtenstein *et al*, 2000).

1.3.2 Initiation and progression

Initiation and progression of prostate cancer are characterized by alterations in regulatory pathways such as apoptosis, androgen receptor (AR) signaling, signal transduction, cell cycle regulation, cell adhesion and cohesion, and angiogenesis (Table 1.3). As discussed in Section 1.2, theses changes occur both within the prostate cells and in the surrounding tissue.

1.3.2.1 Cell cycle regulators

Three cell cycle regulators are of particular interest in prostate cancer:

(i) Ki-67:

Ki-67 protein is a cell cycle-associated nuclear antigen expressed in all phases of the cell cycle except G_0 and early G_1 phases of cells reentering the cell cycle, hence a sensitive biomarker for cellular proliferation (Brown & Gatter, 2002). The Ki-67 proliferation indices for recurrent tumors are approximately double that of the primary tumor (Oomens *et al*, 1991). Increased proliferation index of Ki-67 correlates with the presence of advanced stage disease or increased tumor grade (Cher *et al*, 1995).

Table 1.3: Summary of molecular aberration in prostate cancer (Quinn *et al*, 2005)

Process	**Markers**
Apoptosis	p53, bcl-2
Androgen receptor signaling	Androgen receptor, possible alternate signal transduction pathways
Signal transduction	Epidermal growth factor receptor family
Cell cycle regulation	c-Myc, p16INK4A, p27KIP1, pRb, apoptotic index, Ki-67
Cell adhesion and cohesion	E-cadherin, a-catenin, metalloproteinases, chondroitin sulphate
Angiogenesis	VEGF, VEGF receptors, nitric oxide

(ii) pRb

A close correlation has been established between retinoblastoma gene mutations and the loss of retinoblastoma protein expression (pRb) (Bookstein *et al*, 1990; Geradts *et al*, 1994). Generally, there is progressive loss of pRb expression with increasing prostate cancer grade and stage (Ittmann & Wieczorek, 1996; Phillips *et al*, 1994).

(iii) C-MYC

C-MYC functions primarily as a transcription factor involved in the regulation of apoptosis, cellular differentiation and proliferation (Hayward *et al*, 1981; Hughes *et al*, 2005). It has been reported that C-MYC amplification is present in up to 50% of high-grade prostatic intraepithelial neoplasia (HGPIN) and 73% of primary prostate cancer (Fox *et al*, 1993; Jenkins *et al*, 1997; Qian *et al*,

1997). C-MYC amplification has also been shown to increase with transition through prostatic intra-epithelial neoplasia (PIN) to localized prostate cancer to metastases (Bubendorf *et al*, 1999; Jenkins *et al*, 1997).

1.3.2.2 Apoptosis markers

(i) p53

As discussed in Section 1.2, tumor-suppressor p53 is involved in cell cycle regulation, apoptosis, angiogenesis, and metastasis. Some investigators have compared prostate cancer metastases with primary prostate cancers in the same patients and have concluded that foci with p53 mutations are clonally expanded in metastases (Meyers *et al*, 1998; Stapleton *et al*, 1997; Suzuki *et al*, 1998). p53 nuclear accumulation is increased in metastatic, recurrent and/or androgen-insensitive prostate cancer has also been demonstrated, compared with clinically localized disease (Eastham *et al*, 1995; Grossfeld *et al*, 1998; Navone *et al*, 1993).

(ii) Bcl-2

The bcl-2 oncogene inhibits apoptosis (Haldar *et al*, 1989; Tsujimoto & Croce, 1986). Increased expression of Bcl-2 in prostate cancer has been found to confer androgen resistance, particularly in advanced disease, and may facilitate progression to androgen independence (Apakama *et al*, 1996; McDonnell *et al*, 1992; Raffo *et al*, 1995).

1.3.2.3 Androgen and androgen receptor (AR) signaling

The growth, development and functioning of the prostate is dependent upon androgen, mostly produced by the leydig cells of the testes but also by the adrenal glands (Chatterjee, 2003; Heinlein & Chang, 2004; Santos *et al*, 2004). Recent evidence also supports the production of androgen de novo in the prostate (Mostaghel & Nelson 2008; Locke et al 2008 in press). The main

ligand of AR is 5α-dihydrotestosterone (DHT). DHT binds AR in order to promote cell proliferation, differentiation, and cell death inhibition (Isaacs, 1999). AR is restricted to the cytoplasmic compartment until bound to DHT prompting it to be translocated into the nucleus where it functions as the transcription factor for androgen regulated genes (Sharifi & Farrar, 2006). Since there exists both pre-clinical and clinical evidence supporting retinoblastoma protein, C-MYC, interleukin-4 and 6 and p53 regulation of AR expression (Bernard *et al*, 2003; Grad *et al*, 1999; Lu & Danielsen, 1998), it is conceivable that in selected prostate cancer cells, AR may be amplified, overexpressed through epigenetic regulation and/or contain mutations that allow stimulation by hormones and antiandrogens (Jenster, 1999). It is also suggested that therapy, particularly with non-steroidal anti-androgens, may contribute to selective preferential expression of cells with AR mutations or amplification. Finally, it is believed that, autocrine or paracrine mechanisms may activate pathways to produce downstream AR responses, alone or in collaboration with AR (Jenster, 1999).

1.3.2.4 Tumor-suppressor gene PTEN

PTEN is a tumour suppressor phosphatase that is commonly altered in lethal metastatic prostate cancer (Li *et al*, 1997a; Suzuki *et al*, 1998). PTEN loss occurs in 23% of high grade PIN, and 68% of prostate adenocarcinomas, indicating a role for PTEN in prostate cancer development (Yoshimoto *et al*, 2006). In murine prostate carcinogenesis models, prostate-specific deletion of PTEN resulted in metastatic prostate cancer (Majumder *et al*, 2003; Tolcher, 2004). *In vitro* work shows that reconstitution of PTEN suppresses AR transcription and increases sensitivity to cytotoxic drugs (Huang *et al*, 2001; Nan *et al*, 2003).

1.3.2.5 Prostate cancer progression

Prostate cancer progression to metastasis is believed to involve the following stages: prostatic intraepithelial neoplasia (PIN), followed by adenocarcinoma, then metastasis to secondary sites including bone, lymph node, liver, and lung, with bone being the most predominant site of metastasis (Bagi, 2005). Prostate cancer is considered to be a multifocal and multiclonal disease since patients may have multiple tumors arising independently through different mechanisms such as loss of PTEN (Jin-Tang Dong, 2006; Li *et al*, 1997a; Steck *et al*, 1997).

1.3.3 Prostate cancer diagnosis and treatment

1.3.3.1 Diagnosis of prostate cancer

The digital rectal exam (DRE) and the prostate specific antigen (PSA) tests are used in combination to screen for prostate cancer (Wallace & Powell, 2002). The DRE test involves accessing the prostate via the rectum, in order to determine any structural irregularities indicative of prostate cancer (Wallace & Powell, 2002). The PSA test involves measuring the concentration of PSA, an enzyme secreted by prostatic epithelial cells, in the serum. PSA concentration tends to increase in individuals with prostate cancer due to the enlargement of the prostate (Schwartz *et al*, 2005).

A transrectal biopsy is subsequently carried out when serum PSA levels are greater than 4-10 ng/ml in addition to a positive DRE, suggestive of a cancerous lesion (Wallace & Powell, 2002). Transrectal biopsy would then characterize the potential tumor. Using the Gleason grading system (Gleason & Mellinger, 1974), prostate cancer is given a value of 1-5 based on glandular characteristics, with a lower grade assigned to more differentiated and normal prostate glands, and a higher grade assigned to less differentiated glands. The tumor, node, metastasis (TNM) staging

system is also used by physicians to assess prostate cancer progression. This system indicates that the tumor is restricted within the prostate and surrounding tissues (T), the invasion of the lymph nodes (N), and metastatic spread to distant organs (M) (Hoedemaeker *et al*, 2000). The T stage is subdivided into four groups: T1, T2, T3, and T4. T1 and T2 refer to tumors confined within the prostate while T3 and T4 represent stages where the tumor has invaded the surrounding areas (Figure 1.4). Urologists evaluate Gleason score, TNM staging, and PSA value to predict pathological stages, and determine appropriate treatment recommendations (Ayyathurai *et al*, 2006; Partin *et al*, 1997; Partin *et al*, 2001).

1.3.3.2 Treatment of prostate cancer

Prostate cancer cells depend on androgens for proliferation, protein synthesis initiation and cell death inhibition (Wallace & Powell, 2002). Prostate cancer cells are initially androgen-dependent but a select number develop androgen independency, following medical intervention such as androgen ablation. Although mechanisms which allow prostate cancer cells to survive under very low androgen levels have not been fully elucidated, numerous possibilities have been offered: (i) AR hypersensitivity: cancer cells are able to produce more androgen receptors via amplification of the androgen receptor gene, which allow them to survive under low levels of androgen, and are not androgen independent (Pienta & Smith, 2005). (ii) Androgen are synthesized de novo in the prostate during progression to maintain pre-castrate levels (Locke *et al*, 2008; Mostaghel & Nelson, 2008). (iii) The promiscuous receptor: cancer cells contain mutated androgen receptors that are able to bind and be activated by non-androgen molecules present in circulation and imitating androgen (Debes & Tindall, 2004; Feldman & Feldman, 2001; Nelson *et al*, 2003).

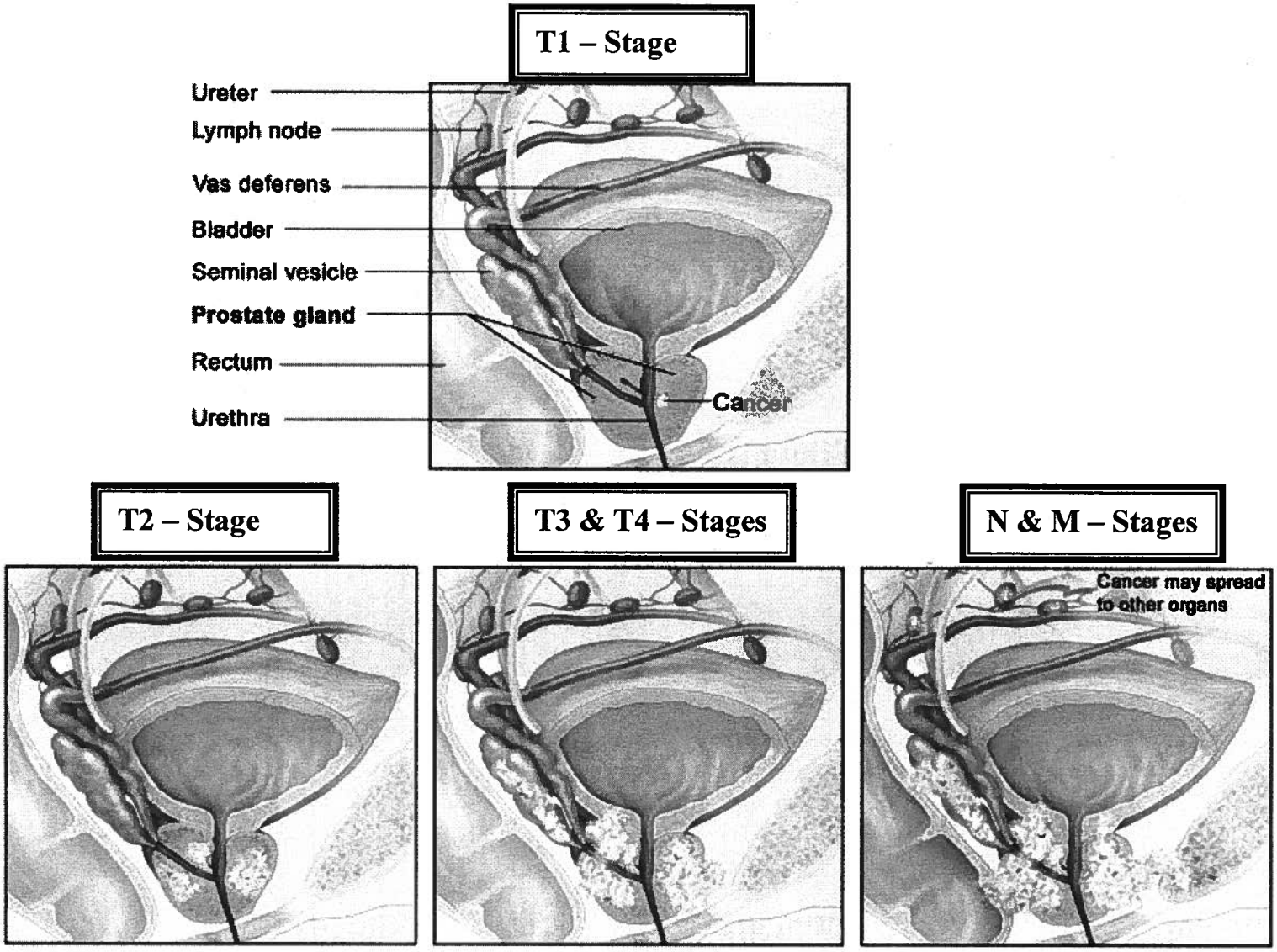

Figure 1.4: Image representing the progression of prostate cancer as described by the Tumor, Node, Metastasis (TNM) staging system (Adapted from U.S. National Cancer Institute PDQ database website (database, 2007). This system indicates that the tumor is restricted within the prostate and surrounding tissues (T), the invasion of the lymph nodes (N), and metastatic spread to distant organs (M).

(iv) Non-cancer cells such as neuroendocrine cells secrete neuropeptides that support the growth of the cancer cells (Pienta & Smith, 2005). Prostate cancer management often involves two stages of treatment: androgen dependence and androgen independence.

(i) Androgen dependence (AD) treatment

In early stage prostate cancer (stages T1 and T2) where the tumor is restricted within the prostatic capsule, the disease is hormone dependent and is readily curable. The two preferred treatment strategies are external radiation therapy and/or radical prostatectomy, the surgical removal of all or part of the prostate gland (Kirby *et al*, 2001). In addition, patients with other co-morbidities may receive "watchful waiting" - observation of the disease without any treatment while the cancer is monitored with subsequent PSA tests and DRE. Androgen ablation therapy (androgen withdrawal) is administered once the cancer has progressed beyond the capsule and/or has metastasized, as prostate cancer cells are androgen dependent (Huggins & Hodges, 1941). Androgen deprivation therapy is a two-step process involving initial administration of luteinizing hormone releasing hormone (LHRH) agonists or orchiectomy, the removal of the testes. This step aims to reduce total circulating androgen levels via reduction of testosterone production. The second step involves administration of AR antagonists, intended to bind AR and this directly inhibit downstream signaling (Tammela, 2004). Nonsteroidal antiandrogens, such as flutamide, nilutamide, and bicalutamide, bind to the AR ligand binding domain, and inhibit activation of the receptor (Farla *et al*, 2005; Furr & Tucker, 1996). Steroidal antiandrogens, such as cyproterone acetate, compete with ligand for receptor binding similar to nonsteroidal antiandrogens, but convey a negative feedback causing suppression of the LHRH axis, thereby reducing testosterone levels (as reviewed by Tammela, 2004; Varenhorst *et al*, 1982). Side effects of androgen ablation treatment include

osteoporosis, fatigue, diminished cognitive function, and loss of libido. Androgen ablation reduces androgen levels significantly but it is believed that it does not eradicate androgen completely.

(ii) Androgen independence (AI) treatment

Despite a 90% primary response rate of androgen ablation therapy, almost all patients advance to a state of androgen independence (AI) with increasing prostate-specific antigen (PSA), and radiologic and/or symptomatic progression (Bhandari *et al*, 2005). In AI prostate cancer, the androgen receptor is reactivated (Zhang *et al*, 2003). Treatment for androgen independent, hormone refractory prostate cancer involves primarily chemotherapy. Docetaxel administered in combination with prednisone constitute the current gold standard chemotherapy for this phase of prostate cancer (Berthold *et al*, 2008; Schurko & Oh, 2008).

1.3.3.2.1 Other natural compounds effective against prostate cancer cells

Docetaxel is derived from a natural product precursor compound and several other natural compounds have been found to be effective in pre-clinical evaluation against prostate cancer cells. The list includes ginsenosides and their metabolites (section 1.4), silibinin and silymarin (flavonoid antioxidants isolated from milk thistle that inhibit the growth of LNCaP cells by causing cell cycle arrest and decrease in PSA levels) (Zi *et al*, 2000), tea polyphenol epigallaocatechin-3-gallate (EGCG, decreases AR mRNA level as well as causes the repression of transcriptional activities of AR promoter region in LNCaP cells) (Ren *et al*, 2000), curcumin (downregulates the transactivation and expression of AR and AR-related cofactors) (Nakamura *et al*, 2002), emodin (a natural compound isolated from *Rheum Palmatum* that inhibits the growth of LNCaP and PC3 stably transfected with AR cells) (Cha et al, 2005), decursin (Jiang *et al*, 2006), inositol hexaphosphate (IP6, a nutrient phytochemical in high-fiber cereals, legumes and nuts, that inhibits the growth of

advanced human prostate carcinoma DU145 cells) (Singh *et al*, 2003), genistein (induces the repression of telomerase activity in DU145 and PC3 cells) (Jagadeesh *et al*, 2006), pomegranate oil (shown to nhibit the growth of DU145 cells by arresting the cells in G2/M phase) (Albrecht *et al*, 2004), oridonin (a diterpenoid from Rabdosia rubescens that induced cytostatic and cytotoxic effects, cell cycle arrest, and apoptosis in LNCaP cells) (Chen *et al*, 2005), and resveratrol (down regulated PSA, the AR co-activator ARA 24 and NF-kB p65 in LNCaP cells) (Narayanan *et al*, 2003).

1.4 Ginseng and ginsenosides

In 1994, 1 million pounds of ginseng was imported into the US where its sale and related products accounted for approximately 300 million US, 1/5 of all botanical sales (Gillis, 1997). This finding reinforces the notion that ginseng is one of the most commonly used plants used in Complementary and Alternative Medicine (CAM), in North America. Ginseng is believed to have been used in Asia for thousands of years but its earliest written reference is included in a herbal medicine compilation called Shennong Bencao Jing (Shennong's Herbal), dated around 502-557 AD (Yun, 2001). The root of ginseng has been reported to have many pharmacological effects including antioxidative and anticarcinogenicity (Nah *et al*, 1995; Yun *et al*, 2001; Zhang *et al*, 1996). Ginseng products have been evaluated in clinical trials for benefits associated with several indicators and conditions, including health-related quality of life (Ellis & Reddy, 2002), erectile dysfunction (Hong *et al*, 2002), postprandial plasma glucose (PG) and insulin (PI) indices (Sievenpiper *et al*, 2004), ability to reverse fatigue (Elam *et al*, 2006), neurodegenerative disorders (Radad *et al*, 2006), and ergogenic properties (Kim *et al*, 2005).

The four main classes of compounds in the ginseng root are: alkaloids, polypeptides, polysaccharides, and ginsenosides. Polysaccharides, which constitute 50- 60% of the ginseng root have been found to possess immunostimulatory activities and have thus been formulated and commercialized as Cold-Fx (CV Technologies, 2008). Cold-Fx was approved by Health Canada with the comprehensive treatment claim of "*helps reduce the frequency, severity and duration of cold and flu symptoms by boosting the immune system*" (Health Canada, 2008). Nonetheless, the principal pharmacologic activities of the ginseng root have been predominantly attributed to ginsenosides (Kim *et al*, 1998; Kitts & Hu, 2000; Lee *et al*, 1997; Yun *et al*, 2001).

Ginsenosides are tri-terpenoid glycoside saponins with a chemical structure consisting of a steroid nucleus to which is bound one or more sugar moieties. Shibata et al. isolated ginsenosides and established their nomenclature according to their Rf (the distance travelled by the sample or analyte divided by distance travelled by the solvent front) values on thin layer chromatography. Thirty five kinds of ginsenosides have been identified thus far, based on the difference in their aglycone (non-sugar structure). These include 22 of 20-(S)-protopanaxadiol type (e.g. Rb1, Rb2, Rc, Rd, Rg3, Rg3, and Rh2), 12 of 20-(S)-protopanaxatriol type (e.g. Re, Rf and Rg1), and 1 of oleanolic acid type (e.g. Ro) (Chan *et al*, 2000; Yun *et al*, 2001). Several of these ginsenosides have been shown to be strong promoters of apoptosis in various cancer models: rat glioma (Kim *et al*, 1999), human melanoma (Fei *et al*, 2002), ovarian cancer (Nakata *et al*, 1998), breast cancer (Oh *et al*, 1999), hepatocellular carcinoma (Park *et al*, 1997) as well as neuroblastoma (Kim *et al*, 2000).

When ginseng is taken orally, Rb_1, Rb_2, and Rc are said to be converted into Rg_3 in the stomach's acidic environment. Bae et al. have shown that Rg3 is in turn transformed into Rh2 under the action of *Fusobactrium* sp. intestinal bacteria. They also demonstrated that a different

group of bacteria, including *Bacteroides* sp., *Eubacterium* sp., and *Bifidobacterium* sp. were capable of further converting Rh2 into 20(S) protopanaxadiol (aPPD) (Bae *et al*, 2002).

1.4.1 Rh2

Rh2 is found in trace amounts within extracts from the roots of *Panax ginseng* C.A. Meyer, and is one of the major *in vivo* metabolic products of ginseng when taken orally. The chemical structure of Rh2 consists of a steroid nucleus and a glucose sugar moiety. Rh2 has been reported to inhibit the growth of and induce anti-metastatic activity in B16 melanoma and ovarian cancer cells (Jia *et al*, 2004; Nakata *et al*, 1998; Wakabayashi *et al*, 1997). In human breast carcinoma MCF-7/MX cells which overexpress breast cancer resistance protein (BCRP), Rh2 significantly enhanced the cytotoxicity of mitoxantrone; an effect that was attributed to inhibition of mitoxantrone efflux and enhanced mitoxantrone uptake (Jin *et al*, 2006). Rh2 was also found to have a G1 phase-specific cell cycle arrest with associated suppression of cdk2 and cyclin E-dependent histone kinase activities apparently as a consequence of upregulation of p21 which binds and inactivates cdk2 (Oh *et al*, 1999). Xie et al. have also shown that Rh2 behaves synergistically with paclitaxel in cultured LNCaP cells, an androgen-sensitive human prostate cancer cell line. In the same study, it was also reported that oral treatment of LNCaP tumors with Rh2 produced a significant decrease in tumor growth which was accompanied with a significant decrease in serum prostate specific antigen (PSA) when combined with intravenously administered paclitaxel (Xie *et al*, 2006). Nakata *et al.* demonstrated that a daily oral administration of Rh2 to nude mice bearing HRA human ovarian cancer xenograft caused an inhibitory effect comparable to a weekly administration of 4 mg/kg of cisplatin (Nakata *et al*, 1998). Like most ginsenosides, Rh2 treated mice showed no signs of toxicity while nearly half of cisplatin-treated mice had to be terminated due to acute body weight

loss. Interestingly, Nakata *et al* attributed the therapeutic effects of Rh2 to enhanced Natural killer cells (NK) activity (Nakata *et al*, 1998). Recently, Wang et al. showed that Rh2 induced cell cycle arrest in the G1 phase and apoptosis in androgen-dependent LNCaP and castrate-resistant PC-3 prostate cancer cells (Wang *et al*, 2008c).

1.4.2 aPPD

aPPD, the aglycone of Rh2, is a gastrointestinally-derived metabolite of *Panax Ginseng* C.A. Meyer as it has been shown that Rh2 could be degraded through deglycosylation to aPPD (Ota *et al*, 1991). It has been suggested that this degradation was observed in the intestine, following oral administration of Rh2 (Xie *et al*, 2005). aPPD has been shown to be a strong promoter of apoptosis (Popovich & Kitts, 2002; Wang *et al*, 2008b), an inhibitor of THP-1 leukemia cell growth, an inducer of DNA fragmentation resulting in build up of sub-G1 cells (Popovich & Kitts, 2002). In human breast carcinoma MCF-7 cells, aPPD significantly enhanced the cytotoxicity of mitoxantrone, inhibited MX efflux, increased mitoxantrone uptake, and inhibited BCRP-associated vanadate sensitive ATPase activity (Jin *et al*, 2006). aPPD has also been established as an inducer of anti-estrogen activity in breast cancer cells (Yu *et al*, 2007) and of pro-apoptosis/autophagy effect in glioma cells (Liu *et al*, 2007). Recently, Wang et al. reported that aPPD also induced cell cycle arrest in the G1 phase and apoptosis in androgen-dependent LNCaP and castrate-resistant PC-3 prostate cancer cells (Wang *et al*, 2008c).

1.5 Development of Drug Combinations

The combination of drugs may lead to therapeutic or pharmacokinetic drug-drug interactions.

1.5.1 Therapeutic drug-drug interaction

Therapeutic outcomes of a combination can be synergistic, additive, or antagonistic. Significantly, the terms synergy, antagonism, and additivity constitute a consensus terminology that emerged from a discussion of experts in pharmacology, toxicology, and biometry (Greco *et al*, 1992). Synergy/antagonism can be defined *as "a greater/lesser pharmacological effect for a two-drug combination than what would be predicted for "no interaction" from the knowledge of the effects of each drug individually"* (Greco *et al*, 1995). This definition makes a "null" or "no interaction" assumption. Two dominant empirical null reference models have been used to establish the foundation of the drug-drug interaction terminology: the Loewe additivity and the Bliss independence models (Bliss, 1939; Loewe & Muischnek, 1926). In the Lowe additivity model, the concentrations (doses) of the drugs in a combination are compared to the concentrations (doses) of the drugs alone that produce the same effect (e.g. 50% inhibition) and the relationship is described by the equation:

$$1 = \frac{D_1}{ID_{X,1}} + \frac{D_2}{ID_{X,2}} \qquad (1)$$

Where D_1 and D_2 are the concentrations of drugs 1 and 2 that yield x% inhibition in the combination. $ID_{x,1}$ and $ID_{x,2}$ are the concentrations of each drug that yield x% inhibition when used alone. The Bliss independence model estimates the effect of the combination of drugs combined

based on their respective effects when used alone. The combination effect estimate is then compared with the observed combination data from the experiment and the relationship is expressed as:

$$fu_{12} = fu_1 fu_2 \quad (2)$$

Where fu_1, fu_2, and fu_{12} are the fractions of possible response (i.e. 50% inhibition) for drug 1, drug 2, and their combination, respectively.

Most methods for assessing therapeutic drug-drug interactions use either the Lowe additivity or the Bliss independence as a null reference model. Methods that use the Lowe additivity include: the isobologram by hand, interaction index of Berenbaum (Berenbaum, 1977), median-effect method of Chou and Talalay (Chou & Talalay, 1984), mutually exclusive model method of Berenbaum (Berenbaum, 1985), bivariate spline fitting (Suhnel, 1990), parametric response surface approaches of Greco et al. (Greco *et al*, 1990) and Weinstein et al. (Weinstein *et al*, 1990), approach of Gessner (Gessner, 1974), parametric response surface approach of Greco and Lawrence (Greco & Lawrence, 1988), and the use of the multivariate linear logistic model (Brunden *et al*, 1988; Carter *et al*, 1986; Carter *et al*, 1988; Carter *et al*, 1983). The Bliss independence model is used as a null reference model by several approaches also including, the fractional product method of Webb (Webb, 1963), the method of Valeriote and Lin (Valeriote & Lin, 1975), the method of Drewinko et al. (Drewinko *et al*, 1976), the method of Steel and Peckham (Steel & Peckham, 1979), and the method of Prichard and Shipman (Prichard & Shipman, 1990).

Although both the Lowe additive and the Bliss independence models are legitimate empirical reference standards for "no interaction", the Bliss independent model has one convincing

argument against its use. Since the Bliss model implies that two agents do not physically or chemically or biologically cooperate, i.e. each agent acts independently of the other, its application is restricted to scenarios where drugs in a combination are known to affect different biochemical sites and not a similar one. This is considered a serious limitation of this model since drug combinations in search of synergy would involve, more often than not, agents with only partially known mechanisms of action. The use of the Bliss model would then be relegated for agents with well-defined mechanisms of action only. This limitation of the Bliss model presents the Lowe additivity as a superior model (Greco *et al*, 1995). In that perspective, approaches that use the Bliss independence as the "no interaction" model for assessing therapeutic drug-drug interactions are subject to the same disadvantage. Amongst approaches using the Lowe additivity model, the isobologram by hand is the simplest and least expensive. This approach is similar to the interaction index calculation of Berenbaum (Berenbaum, 1977), requiring only equipments such as the pencil, ruler, and graph paper. The drawback of these approaches includes excessively time-consumming to plot. In addition, they lack an obvious summary measure of the intensity of interaction, an objective statistical procedure, and they require that the data range encompasses the IC_x level. The mutually exclusive model method of Berenbaum (Berenbaum, 1985) is a computer similated version of the interaction index calculation of Berenbaum. Accordingly, although superior, its most significant advantage is a lack of an obvious summary measure of the intensity of interaction. Other approaches using the Lowe additivity model include both non-parametric (does not require an a priori assumption) and parametric (requires an a priori assumption) approaches. Non-parametric approaches such as the Bivariate spline fitting (Suhnel, 1990) and the method of Prichard and Shipman (Prichard & Shipman, 1990), although very flexible, have the disadvantage of being mostly exploratory approaches and require a large number of regularly dispersed points for fitting

with bivariate splines (a special functions defined piecewise by polynomials, in the case of the Bivariate spline fitting method). Parametric approaches include models of Greco et al. (Greco *et al*, 1990) and Weinstein et al. (Weinstein *et al*, 1990). Contrary to other approaches presented thus far, parametric models are rigorous and provide a quantitative measure of the intensity of interaction, a measure of its uncertainty, and an objective statistical procedure. Their main disadvantages include the requirement for advanced statistical and computing expertise and the fact that different parametric models sometimes lead to different conclusions from the same data set. The median-effect method (MEP) of Chou and Talalay (Chou & Talalay, 1984) is arguably the most influential of all drug interaction assessment methods introduced since 1970. MEP was developed from the law of mass action and an enzyme kinetic model (Chou & Talalay, 1984). The MEP equation is represented by

$$\frac{f_a}{f_u} = \frac{D}{(D_m)^m} \tag{3}$$

Where f_a is the fraction affected by the drug, f_u is the unaffected fraction (1-fa), D is the dose of the drug, Dm is the median-effect dose (signifies drug potency) and m is an exponent signifying the shape of the dose-effect curve. This equation determines whether the effect of a drug combination is greater than expected (synergistic) based on the effect of the single agents. The MEP defines also the combination index (CI) function, calculated using

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \tag{4}$$

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_1(D)_2}{(D_x)_1(D_x)_2} \tag{5}$$

Where D_1 and D_2 are the doses of drug 1 and drug 2. D_x refers to the dose of drug required to produce an effect of x percent. Equation 4 and 5 apply to mutually exclusive and mutually nonexclusive drugs respectively. In a drug combination, CI < 1, CI > 1, and CI = 1 indicate synergy (a more than expected additive effect), antagonism (a less than expected additive effect), and additivity, respectively. The MEP is also used to obtain the Dose Reduction Index (DRI), a measure of the extent to which the dose of each drug, in a synergistic combination, is potentially reduced at a given effect level (IC_{50}) compared with the doses for each drug alone.

$$DRI = \frac{D_x}{D_c} \tag{6}$$

Where D_x is the single drug dose required to produce an effect of x percent (50%). Dc is the dose of the drug in a combination that generates the same effect.

The most important advantages of the MEP includes the consideration that contrary to the other approaches, its fundamental equations were developed from basic mass action enzyme kinetics, thereby qualifying it as the only approach where estimated parameters have the potential of being biologically meaningful. In addition, the MEP uses an established statistical approach, the linear regression, and requires fewer data points compared to the other methods. The availability of the MEP of Chou and Talalay in affordable software also contributes to its wider use. Two key disadvantages of the MEP approach have been reported: (i) Other investigators have convincingly showed that the CI equation for the mutually nonexclusive model (Equation 5) was slightly inaccurate (Lam *et al*, 1991; Syracuse & Greco, 1986) and led to artifacts in the CI vs fa plot. (ii) There is a lack of practical guidance from Chou and Talalay for $fa \leq 0$ or $fa \geq 1$. The MEP software

does not accommodate these values but decision to modify them is left to the investigator despite the fact that it critically affects the final result.

In conclusion, the MEP method of Chou and Talalay appears to be the only relevant method for biological applications since it was derived from mass action enzyme kinetics. In addition to using an established statistical approach, it is the most convenient and accessible approach considering that it requires very few data points to be robust and its software is both affordable and practical in its use.

1.5.2 Pharmacokinetic drug-drug interaction

Drug-drug interactions may affect the pharmacological behavior of the drugs involved in several ways including: (i) alteration of the serum concentration, (ii) alteration of hepatic metabolism due to induction or inhibition of hepatic enzymes by drugs such as Phenobarbital (inducer) and cimetidine (inhibitor), (iii) inhibition of cytochrome P450 enzymes by drugs such as quinidine and ketoconazole (Health Canada, 1994), and (iv) inhibition of drug transporters. It can be inferred that the most important known drug-drug interactions involve the modulation of drug metabolism and transport processes. Significantly, the U.S. Food and Drug Administration (FDA) requires that the metabolism of an Investigational New Drug (IND) be defined during drug development and that its interactions with other drugs be explored as part of an adequate assessment of its safety and effectiveness (FDA, 1999).

1.5.2.1 Cytochrome P450-mediated drug-drug interactions

The blood concentration of an administered drug is affected by its absorption, distribution, metabolism and/or excretion. Elimination of a drug or its metabolites occurs by metabolism or

excretion. Although numerous drug-metabolizing enzymes are involved in metabolic drug interactions, the cytochrome P450 family of enzymes (CYP) appears to be the most prominent player (Guengerich, 1997; Lin, 2006; Parkinson, 1996). Studies in laboratory animals demonstrate that CYP is present in various tisues with its highest level in the hepatic endoplasmic reticulum and the small intestine mucosa (Hardwick *et al*, 1983; Leighton & Kemper, 1984; Taniguchi *et al*, 1980). It is established that three members of the CYP family play a significant role in drug metabolism in the vertebrates, namely CYP1, 2, and 3, all highly expressed in the liver but also present in other tissues (Ding & Kaminsky, 2003; Lin & Lu, 1998). CYP1 which is also significantly present in the lung, small intestine, and placenta are involved in the metabolism of polycyclic aromatic hydrocarbons, heterocyclic compounds, methylxanthines, and aromatic amines (Gonzalez *et al*, 1992). CYP2 and 3 are also present in high concentration in the small intestinal epithelium. They are involved in the metabolism of various organic chemicals, drugs, and steroids, including the 6β-hydroxylation of testosterone (Crespi *et al*, 1989; Fuhr *et al*, 1992a; Fuhr *et al*, 1992b; Song *et al*, 1986; Umeno *et al*, 1988). CYPs are known to catalyze more than forty different types of reactions, including hydroxylations but also including deaminations, desulfurations, dehalogenations, epoxidations, N-, S-, and O-dealkylations, N-oxidations, peroxidations, and sulfoxidations (Coon *et al*, 1996; Danielson, 2002; Omura, 1999).

It is estimated that the metabolism of more than 50% of all drugs known to be metabolized by the CYP family is carried out by the CYP3A sub-family (Benet, 1996). CYP3A members include CYP3A4, 5, and 7. Of all CYP3A family members, CYP3A4 is the most prominently expressed in the liver, with an average of 29% of the total P450 concentration (Shimada *et al*, 1994). The activity of CYP3A4 has been shown to be competitively inhibited by ginsenoside

Rh1(Liu *et al*, 2006a), and ginseng intestinal bacterial metabolites compound K, aPPD, and aPPT (Liu *et al*, 2006b).

1.5.2.2 Drug transporters modulation of drug-drug interactions

P-glycoprotein (P-gp), which is encoded by the multidrug resistance 1 gene (MDR1), and the multidrug resistance-associated protein 1 (MRP1), are two members of the mammalian superfamily of ATP-binding cassette (ABC) transporters which mediate the movement of various substrates including simple ions, complex lipids and xenobiotics. One of the most common reasons for tumor cells developing a multidrug resistant phenotype is the overexpression of P-gp or MRP1, both capable of moving drugs and other substances through a cellular efflux against a concentration gradient using the energy of ATP hydrolysis (Lepper *et al*, 2005).

The ABC gene products, including ABCB1 (P-glycoprotein) and ABCG2 (also known as breast cancer-resistance protein, BCRP; mitoxantrone-resistance protein, MXR; or ABC transporter in placenta, ABCP) influence oral absorption and disposition of a wide variety of drugs. As a result, the expression levels of these proteins in humans influence an individual's susceptibility to certain drug-induced side effects, interactions, and treatment efficacy (Lepper *et al*, 2005). P-gp is expressed in many cells lining the luminal surface of several tissues known for their excretionary and barrier functions, such as the liver, kidney, small intestine, the blood-brain barrier, and the blood-testes barrier (Thiebaut *et al*, 1987; Thiebaut *et al*, 1989). In chemotherapy, co-administration of cytotoxic anticancer drugs with known P-gp modulators are used to increase the therapeutic effectiveness of these drugs. This co-administration alters the pharmacokinetics of the chemotherapeutic drugs and contributes to overcoming drug resistance. Significantly, in women with ovarian cancer, clinical resistance to paclitaxel, a P-gp substrate, was overcome in

approximately 20% of the cases with the addition of PSC 833, a highly effective P-gp modulator (Oza, 2002). Verapamil, the calcium channel blocker, was one of the first compounds observed to be a P-gp inhibitor based on its ability to increase the sensitivity of murine leukemia cells to vinblastine (Tsuruo *et al*, 1981). More recently, Kim et al. showed that ginsenoside Rg3 inhibited P-gp expression and reversed P-gp-mediated multidriug resistance in the multidrug-resistant human fibroblast carcinoma cells (Kim *et al*, 2003).

1.5.3 Benefits of drug combination in cancer treatment

Clinical evidence demonstrates a correlation between the number of drugs used in a combination regimen and the percent of complete response and cure rate (Frei & Antman, 1997). Combination therapy offers great benefits in comparison to single-agent therapy by providing: (i) Maximal cell kill within the range of tolerable toxicity; (ii) Diverse interactions between the drugs and the heterogeneous tumour; and (iii) The potential to prevent or slow the subsequent development of cellular drug resistance. Combination regimens of chemotherapeutic drugs were developed in the 1960s and early 1970s, following the establishment that single drug administration at clinically tolerable dosages failed to cure cancer. These drug combinations were based on biochemical actions of the individual drugs but still remained mostly ineffective in curing cancer. It is now suggested that drug selection for the most effective drug combinations should comply with the following principles: (i) Individual drugs should be partially effective against the same tumour, (ii) Toxicity of individual drugs should not overlap, (iii) Drugs should be used at their optimal dose and schedule; and (iv) The biochemical, molecular, and pharmacokinetic interactions of individual drugs in the combination should be investigated (Chu & Devita, 2005). In addition to the aforementioned benefits, combination therapy may involve an effective drug efflux transporter

inhibitor and its therapeutically effective substrate. The inhibition of efflux transporters may lead to chemosensitization and reversed multidrug resistance (Litman *et al*, 2001; Perez-Tomas, 2006).

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2. FIRST MANUSCRIPT CHAPTER

Pre-clinical evaluation of Rh2 in PC-3 human xenograft model for prostate cancer *in vivo*: Formulation, Pharmacokinetics, Biodistribution and Efficacy[1]

[1] A version of this chapter has been submitted for publication. Co-authors: Andy Eberding, Catherine A. Wood, Hans Adomat, Ladan Fazli, Antonio Hurtado-Coll, William Jia, Ph.D, Marcel B. Bally, and Emma S. Tomlinson Guns.

2.1 Introduction

Prostate cancer is the fifth most common neoplasm worldwide, and the second most common cancer among men. Of all new cancer cases in 2002, 679,000 of them were prostate cancer which resulted in 221,000 deaths (Parkin *et al*, 2005). Surprisingly, there are really no chemotherapeutic drugs that are considered highly effective when used to treat prostate cancer patients that have relapsed and/or have metastatic disease. In recent years, however, new drugs and drug combinations and particularly those involving taxanes have contributed to improvements in treatment outcomes in prostate cancer (De Wit, 2008; Oh, 2003). It has been suggested that optimal drug combination effects will be achieved when combining drugs which have different mechanisms of action and different toxicity profiles. In addition, drug combinations that interact in a manner that result in synergy or drug potentiation (i.e. where the combined effects are far greater than that expected on the basis of the effects of the individual agents) are of great interest. This latter class of drug combinations is of particular interest since many highly specific targeted agents may not exhibit sufficient activity when given as a single agent, but when used in combination may provide meaningful therapeutic outcomes. This is also becoming of interest in the context of complementary medicines, agents that are being used by patients with the belief that they may augment treatment responses achieved by conventional therapy.

In a recent comprehensive study, it was concluded that 83% of cancer patients attending the MD Anderson Cancer Center in Texas acknowledged using complementary and alternative medicine (CAM). Their main reasons for using CAM included its assumed reputation for apparent lack of toxicity (Richardson *et al*, 2000). Research is required to delineate this assumption, but one working assumption is that combinations of non-toxic effective

phytochemicals with established chemotherapy agents could offer the potential of enhanced drug efficacy without adding to the toxicity of the conventional drug. We have a particular interest in assessing the therapeutic potential of Rh2 (Figure 2.1A); a biologically active phytochemical extracted from the roots of *Panax ginseng* C.A. Meyer. Rh2 is a tri-terpenoid glycoside saponin with a chemical structure consisting of a steroid nucleus and a glucose sugar moiety. Rh2 has a 20(S)-protopanaxadiol dammarane skeleton aglycone type and has been reported to inhibit the growth of and induce anti-metastatic activity in B16 melanoma and ovarian cancer cells (Jia *et al*, 2004; Nakata *et al*, 1998; Wakabayashi *et al*, 1997). In human breast carcinoma MCF-7/MX cells which overexpress

breast cancer resistance protein (BCRP), Rh2 significantly enhanced the cytotoxicity of mitoxantrone; an effect that was attributed to inhibition of mitoxantrone efflux and enhanced mitoxantrone uptake (Jin *et al*, 2006). Rh2 was also found to have a G1 phase-specific cell cycle arrest with associated suppression of cdk2 and cyclin E-dependent histone kinase activities apparently as a consequence of upregulation of p21 which binds and inactivates cdk2 (Oh *et al*, 1999). Although encouraging, further development of Rh2 requires a drug development strategy that is similar to that of any new anticancer agent. In this regard, issues related to drug formulation, analysis, stability as well as therapeutic activity alone and in combination with existing standard of care drugs need to be systematically characterized prior to development of clinical studies assessing therapeutic activity in patients. Recently, Wang et al. investigated possible mechanisms responsible for the antiproliferative, pro-apoptotic, and cell cycle arrest effects of Rh2 against LNCaP androgen-sensitive and PC-3 androgen-insensitive prostate cancer cells. They showed that Rh2 increased the expression of the cdk inhibitor p21 and tumor suppressor protein p53 while decreasing the levels of cdks 2, 4, and 6 (Wang *et al*, 2008). They

also investigated the *in vivo* efficacy of 25-OCH_3-PPD, a ginsenoside aglycone, in combination with docetaxel, both administered intraperitoneally.

We have previously shown that Rh2 and paclitaxel acted synergistically in cultured LNCaP cells, an androgen-sensitive human prostate cancer cell line (Xie *et al*, 2006). In the same study, we also reported that oral treatment of LNCaP tumors with Rh2 produced a significant decrease in tumor growth which was accompanied with a significant decrease in serum prostate specific antigen (PSA) when combined with intravenously administered paclitaxel (Xie *et al*, 2006). The present study is the first in a series of investigations exploring the pre-clinical development and efficacy testing of Rh2 in an androgen-insensitive model for prostate cancer. Rh2 is thus being evaluated as a potential agent for use in combination with docetaxel for treatment of locally advanced and metastatic prostate cancer. Docetaxel and Rh2 are administered intravenously and orally, respectively; two relevant pharmacological routes of administration. Initially, the solubility and stability of Rh2 in ethanol was determined. The ethanol solubilized drug was formulated for oral gavage with propylene glycol and water. A high performance liquid chromatography tandem mass spectroscopy (LC/MS) method was then developed and validated for the quantitation of Rh2. This assay was used to determine the pharmacokinetic and biodistribution of Rh2 following oral administration to nude mice. The dose used for these preliminary pharmacokinetic studies was determined to be well tolerated and effective when used to treat animals bearing established PC-3 tumors, an androgen-insensitive human prostate cancer model. Serum creatinine levels, as a measure of renal toxicity, were measured using a published HPLC method (Dunn *et al*, 2004). Liver toxicity was assessed by measuring serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

levels (Wang *et al*, 2007). Antitumor activity was associated with a increase in apoptotic cells and a decrease in the proliferation marker Ki-67 (Kameyama *et al*, 2000).

2.2 Materials and methods

Test compounds and materials

Ginsenoside Rh2 (M.W. 640.89 g/mol), as a white powder, was purchased from LKT Laboratories Inc., St. Paul, MN. (USA), and generously supplied by Panagin Pharmaceuticals Inc, Richmond, BC (Canada). Taxotere® (docetaxel) was purchased from BC Cancer Agency Pharmacy as a 40 mg/ml solution in polysorbate 80, manufactured by Aventis Pharma Inc. Saint-Laurent, Québec (Canada). Cholic acid (M.W. 408.58 g/mol) and all other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

LC/MS analysis of Rh2

Instrumentation and conditions

The HPLC system consisted of an integrated Waters Alliance 2695 quarternary solvent delivery system with low pressure mixing, plus autosampler and 996 photodiode array detector (Waters Corp., Milford, MA). Rh2 and cholic acid (internal standard) were resolved on an Exterra C18 column (3.5 μm, 50 mm x 2.1 mm; Waters, Milford, Massachussets) with Mobile Phases A, B, C, and D; ddH_2O, methanol, acetonitrile, and 0.5% acetic acid respectively. The following gradient profile was used: t = 0-2 min, 40% A, 50% B, 10% D; t = 3-6 min, 10% A, 70% B, 10% C, 10% D, t = 7 min, 70% B, 20% C, 10% D; t = 8 min, 50% B, 40% C, 10% D, t = 8.5-9.0 min, 50% B, 50% C; t = 10-14 min, 100% C; t = 15-22 min, 40% A, 50% B, 10% D. The flow rate was 0.2-0.25 ml min^{-1}. A Waters/Micromass ZQ 2000 detector was used for quantitative analysis of Rh2 and cholic acid. The ZQ 2000 detector settings were as follows: Capillary: 3kV, Desolvation temperature: 300°C, Source temperature: 120°C, Cone temperature: 20°C, Extractor, RF lens and Multiplier voltages: 1.0, 0.5 and 650 V respectively, desolvation and Cone gas flows: 350 and 50 l/hr respectively. For Single Ion Recording (SIR) functions, *m/z*

values of 681.0 and 407.2 were selected for Rh2 and cholic acid respectively, with 0.1 sec dwell and a cone voltage of 50 V. MassLynx version 3.4 (Micromass UK Ltd, Manchester, UK) was used to perform peak integration.

Assay validation

Primary standard solutions of Rh2 were prepared in 100% ethanol and spiked with cholic acid (internal standard). Secondary standard solutions were obtained by spiking 120 µl of 95% methanol with 10 µl of the primary standard solution to yield nominal concentrations over a range of 0.01 – 20.0 µg/ml. All standard solutions were stored at 4°C.

Linearity and range

Five concentrations of Rh2 standard solutions were analyzed (n=3): 0.5, 2.0, 5.0, 10.0, and 20.0 µg/ml. The minimum acceptable correlation coefficient to establish linearity was set at 0.99. The detector response was correlated against analyte concentration by least-squares regression.

Precision and accuracy

Three concentrations of Rh2 standard solutions were analyzed (n = 6) over 3 days: 0.5, 2.0, and 10.0 µg/ml. The mean, standard deviation (S.D.), coefficient of variation (C.V.), and relative error (R.E.) were determined for the six replicate runs.

Limit of detection (LOD) and limit of quantitation (LOQ) of Rh2

Stock solutions of Rh2 were prepared in triplicate at 12 concentrations: 0.001, 0.02, 0.05, 0.5, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, and 500.0 µg/ml. We defined the LOD as the minimum concentration that could be detected based on a signal-to-noise (S/N) ratio of 3 to 1. The LOQ was the minimum concentration that could be quantified based on S/N ratio of 10 to 1.

Solubility

Standard solutions of Rh2 in ethanol were prepared between 1 and 10 μM (1 μM, 2.5 μM, 5 μM, 7.5μM, and 10 μM) and analyzed by HPLC. A calibration curve of peak area versus concentration was generated and a linear relationship shown. Three sets of saturated solutions of Rh2 were then prepared at room temperature by incubating an excess of the compound in 2 ml of 100% ethanol in capped test tubes. The mixture was vigorously mixed and stored at room temperature for 30 minutes; this time was determined to be sufficient for insoluble materials to settle from the mixture. The clear supernatant was sequentially diluted and the amount of Rh2 in the diluted solutions quantified by HPLC. Using the calibration curve linear equation and incorporating dilution factors, the amount of compound dissolved in the saturated solutions was determined.

Stability of samples at 4°C, room temperature, and 60°C

232 mg of Rh2 was dissolved in 1.8 ml of 100% ethanol, a concentration based on the maximum solubility of 130 mg/ml determined using the methods described above. The mixture was mixed vigorously and 200 μl pipetted into each of nine eppendorf tubes. One set of three eppendorf tubes was stored at 4°C (n = 3), the second set of tubes (n = 3) at room temperature, and the last set (n = 3) at 60°C. The concentration of Rh2 in these solutions was determined by HPLC on day 1, 2, 3, 7, 14, 21 and 28.

Oral gavage formulation

232 mg of Rh2 was dissolved in 1.8 ml of 100% ethanol and mixed vigorously (based on maximum solubility of 130 mg/ml). In a separate container 700 μl of ddH_2O was mixed (by

vortexing) with 5.5 ml of propylene glycol. The propylene glycol-water mixture was then mixed with the Rh2-containing ethanol solution.

Pharmacokinetic and biodistribution studies

PC-3 cells (1.0×10^6) were inoculated subcutaneously into 6-8 week old male nude mice (Harlan Sprague Dawley, Inc.) weighing 25-31 g. After 14 days, the developing tumors were measured and mice randomly assigned in different treatment groups based on their weight. Ten groups of three mice were dosed by oral gavage with Rh2 at 120 mg/kg (103-128 µl) (highest achievable dose, limited due to gavage volume limitations (150 µl) imposed by the institutional animal care committee) or the vehicle control at an equivalent volume based on weight. Mice were terminated using CO_2 asphyxiation upon which blood (obtained by cardiac puncture) and major tissues (stomach, small intestine, liver, kidney, spleen, brain, and lung) were promptly collected at 30, 60, 120, and 200 minutes after administration. Typically, 500 to 700 µl of blood was obtained and then placed into a Plasma Separator tube (Microtainer®, Becton Dickinson, NJ, USA), mixed, and placed on ice. The prostate and tumors were then collected at 10, 20, 30, 45, 60, 90, 120, 150, and 200 minute time points. Tissues were collected and flash frozen, prior to placing the samples in storage at –80°C. To assess Rh2 extraction efficiency, blank plasma and tissue homogenate samples (obtained from vehicle treated mice) were spiked with Rh2 and cholic acid to achieve three concentrations (0.5, 2.0, and 10.0 µg/ml). The spiked samples were subsequently extracted and analyzed using the HPLC method described above.

Plasma – Solid-phase sample preparation

C18 Sep-Pak extraction columns mounted on a vacuum extraction manifold (Waters, Massachusetts, USA) were first equilibrated with 1 ml of 100% methanol and 1 ml of ddH_2O.

After loading with 200 µl of plasma spiked with 10 µl of cholic acid (internal standard), each column was subsequently washed with 1 ml of ddH_2O followed by 1 ml of 30% methanol. The column was then eluted twice with 1 ml of 100% methanol into eppendorf tubes, and the eluate was dried in a speed-vac (Labconco Centrivap) at 35°C. The residue was reconstituted with 100 µl of 100% methanol, mixed vigorously, sonicated and transferred into HPLC auto-sampler vials. A non-compartmental method using the nonlinear least squares regression program WinNonlin (Scientific Consulting Inc., Cary, N.C., USA) was used to analyze the plasma concentration data at four time-points: 30, 60, 120, and 200 min. The area under the plasma concentration versus time curve (AUC) was calculated using the trapezoidal rule extrapolated to infinity. Pharmacokinetic parameters were generated including the terminal half-life ($t_{1/2}$) (n = 3), the systemic clearance (Cl), the peak plasma concentration (C_{max}), and the time to reach C_{max} following oral administration (T_{max}).

Tissues

Tissue samples were ultrasonicated in 4 x w/v of 0.01 M pH 7.8 phosphate buffer. 200 µl of the homogenate was spiked with cholic acid (internal standard) and mixed for one minute. Ice cold acetonitrile (800 µl) was added to the homogenate and mixed for one minute for protein precipitation. The sample was then centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was dried at 35°C, the residue was reconstituted in 200 µl of 100% methanol, vortexed and sonicated, and injected onto the HPLC column. Standard calibration curves were analyzed at three concentrations (n = 3): 0.5, 2.0, and 10.0 µg/ml.

Efficacy and Toxicity of Rh2

PC-3 cells (1.0×10^6) were inoculated subcutaneously into 6-8 week old male nude mice (Harlan Sprague Dawley, Inc.) weighing 25-31 g. After 14 days, the developing tumors were measured and mice randomly assigned in different treatment groups based on tumor size. n = 8 for the Rh2 and docetaxel groups while n = 6 for the oral gavage control and saline groups. A caliper was used to measure the three perpendicular axes of each tumor. The formula $V = (L \times W \times H)\frac{\pi}{6}$ where L is the length, W the width, and H the height, was used to calculate the tumor volume. Mice bearing measurable tumors qualified for the study and the average tumor volume when treatment was initiated was 120 mm^3. Mice were weighed five days a week and tumors measured twice a week for 25 days. Four different treatment groups were defined: (i) Rh2 solubilized in ethanol:propylene glycol:water (2:7:1 ratio) was administered by oral gavage at a dose of 120 mg/kg (103-128 µl volume range) using a QD x 5 schedule each week for 4 weeks; (ii) vehicle control consisting of ethanol:propylene glycol:water (2:7:1 ratio) given at an equivalent dose of 120 mg/kg (103-128 µl volume range) using a QD x 5 schedule each week for 4 weeks; (iii) docetaxel, used as a positive treatment control, was administered intravenously via the lateral tail vein at a dose of 20 mg/kg (100-124 µl volume range) using a Q7D x 4 dose schedule; and (iv) saline (100-124 µl volume range) given Q7D x 4. The mice were monitored daily for changes in weight and other signs of acute toxicity. Serum creatinine levels were determined using an HPLC system consisting of a Waters 2695 separations module paired with a Waters 996 photodiode array detector (Waters Corp., Milford, MA). Creatinine was resolved on a 3.0 µm bore diameter 2.1 x 50 mm Waters Atlantis™ HILIC Silica column with a Mobile Phase consisting of 95 % acetonitrile solution containing 0.025% ammonium formate. Serum ALT and AST were quantified using standard ALT and AST kinetic assay kits (Stanbio

Laboratory, Boerne, Texas) adapted in 96-well plates. For serum creatinine, ALT and AST level determination, Rh2 treated mice were compared to the control and the untreated groups.

Analysis of apoptosis and proliferation markers

At the end of the efficacy studies, mice were sacrificed and tumors were excised, formalin-fixed, and then paraffin-embedded. A Tissue Micro-Array (TMA) was constructed by extracting four 600-μm diameter cores of tumor tissue from each paraffin block using a Beecher Instruments tissue core extractor and re-embedding these cores into a gridded paraffin block. After construction, 4-μm tissue sections were cut and adhered to Fisher SuperFrost Plus glass slides. Apoptotic cells were then visualized by terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining which was carried out using the ApopTag® Peroxidase Kit. Tissues were pretreated with proteinase K for 15 minutes. Subsequently, TdT enzyme was applied and the tissue incubated in a humid chamber at 37°C for 1 hour. Anti-digoxigenin peroxidase conjugate was later applied for 30 minutes, followed by DAB, and counter-stain with hematoxylin, as well as Staining Blueing Reagent. For Ki-67, a Mouse-To-Mouse immunohistochemical detection kit (Chemicon®) was used to decrease nonspecific staining. Ki-67 (Dako, Carpinteria, California) (1:200) monoclonal antibodies were used in combination with immunoperoxidase procedures (LSAB + peroxidase kit). Antigen retrial was applied by steaming with citrate buffer for 30 minutes. The TMA slides were imaged digitally and evaluated by visual scoring of apoptotic and Ki-67 positive cells. Scores from individual tumor cores with significant necrosis were omitted. Scoring was completed by two pathologists blinded to the study groups.

Statistical analysis

For each studied variable, mean and standard error of the mean (S.E.M.) were calculated. Statistical significance and differences between the described treatment groups were assessed using the Tukey-Kramer/Student's t-test from within One-way Analysis of Variance (ANOVA) analysis. For these tests, the level of significance was set at a p value of at least < 0.05. All mean values were reported as mean ± S.E.M.

2.3 Results

Assay validation

The limit of detection (LOD) and the limit of quantitation (LOQ) of Rh2 were measured using the LC/MS method described in the Methods section. Using this assay the estimated LOD was 2.0 ng/ml while the LOQ that can be reliably and reproducibly measured was estimated to be 5.0 ng/ml. Standard curves for Rh2 showed linearity over the selected concentration range (0.01 – 20.0 μg/ml). The correlation coefficients were > 0.99 throughout the validation procedure. Inter-day precision and accuracy as expressed by the RSD and the C.V. and R.E. respectively were less than 10%.

Solubility, stability and formulation in the ethanol-propylene glycol-water ternary solvent system.

In order to formulate Rh2 for oral gavage, a strategy based on the use of the ethanol-propylene glycol-water ternary solvent system was developed. The first step in this process was to assess solubility of Rh2 in 100% ethanol at room temperature. The maximum solubility of Rh2, as determined by LC/MS analysis of the supernatant of saturated solutions, was 130.0 ± 2.3 mg/ml. Ethanol solutions of Rh2 were stored at 4°C, room temperature and 60°C over a time frame of one month to assess Rh2 stability. The results, summarized in Figure 2.1B, suggest that Rh2 is stable at 4°C and room temperature. LC/MS analysis of Rh2 indicated that the drug concentration did not change over the 4 week time period. Rh2 underwent rapid thermal degradation, measured as a loss of intact drug, at 60°C. An approximate decrease of 40% in parent drug concentration was observed within 2 days and the drug was not detectable by day 28 (Figure 2.1B). The rate constant for the observed decrease in concentration at 60°C, k (60°C), is 0.14 and the representative compound half-life, $t_{1/2}$ (60°C) was estimated to be 12.5 days. A

concentration change of ± 15% is generally considered acceptable in stability assays (USP, 2000) therefore it was concluded that ethanol solubilized drug solutions could be prepared and stored at 4°C for at least a time frame appropriate for the toxicity and efficacy assessments summarized below. Using the ethanol-propylene glycol-water ternary solvent system diagram designed by Sorby *et al.*(Sorby *et al*, 1963), the dielectric constant of the ethanol, propylene glycol and water mixture combined at the 2:7:1 v/v ratio was estimated to be approximately 31. The maximum solubility of Rh2 was achieved at this 2:7:1 v/v ratio.

Pharmacokinetics and Biodistribution

Extraction efficiencies for Rh2 from mouse plasma and tissues at three different concentrations (0.5, 2.0, and 10.0 μg/ml) were determined as described in the Methods and the results summarized in Table 2.1A. The extraction efficiency from plasma was 65.2 ± 2.4 % and this was not influenced by the concentration of Rh2 used in these assays. Extraction efficiencies from tissues varied from 79.6 ± 3.2 % in stomach to essentially 100% in the kidney. Tissue standard calibration curves, summarized in Figure 2.2, exhibited excellent linearity (correlation coefficients of 0.98 or greater).

The plasma levels of Rh2 determined at various time points following oral administration of Rh2 have been summarized in Figure 2.1C. Rh2 levels in the plasma peaked at 19 μg/ml and after 200 minutes were below the LOQ. Using these data, the pharmacokinetic parameters for Rh2 were estimated and summarized in Table 2.1B. The half-life ($t_{1/2}$) was 99.6 ± 6.6 min., the clearance (Cl_{oral}) was 46.5 ± 2.7 ml/min/kg while T_{max} was 30.0 min. The AUC was 1884.3 ± 209.2 μg.min/ml while C_{max} was 19.0 ± 2.0 μg/ml.

Tissue distribution data have been summarized in Figure 2.3. As expected, given the route of administration, Rh2 levels appeared to be highest in the small intestines, with approximately

54% of the drug recovered in the small intestines 30 minutes after the gavage was administered. Approximately 27% of the administered dose is found in the liver at the same time point. Rh2 level was not quantifiable in the brain at any of the selected time points and all tissues exhibited Rh2 levels below the LOD 200 minutes after administration. Approximately 0.08% of the administered dose was found in the prostate after 120 minutes and the Rh2 levels in this tissue remained quantifiable 200 minutes after administration (Figure 2.3E). The percent of administered dose localizing in the PC-3 tumor tissue was at 0.3% 90 minutes following administration (Figure 2.3F). The results clearly suggest that Rh2 is bioavailable after oral gavage in the vehicle formulation.

***In vivo* efficacy and toxicity**

The therapeutic activity of Rh2 was determined using mice bearing tumors established following subcutaneous injection of PC-3 human prostate cancer cells. This study included negative control groups (saline and the vehicle control) as well as a positive treatment control (docetaxel administered Q7D x 4 at an established therapeutic dose (maximum tolerated dose (MTD)). The results summarized in Figure 2.4A clearly demonstrate that Rh2 was effective in inhibiting PC-3 tumor growth. At the end of the study (39 days after tumor cell inoculation, 25 days after treatment was initiated), the average tumor volume for saline treated and vehicle control treated animals was approximately 3 times the size of the average tumor volume determined at the time treatment was initiated. For animals treated with Rh2 the tumor volume did not change during this time course. Rh2 treatment group was statistically different from the control (p=0.012) and saline (p=0.008). Lack of tumor progression in animals treated with Rh2 is consistent with the belief that this ginsenoside will exert cytostatic effects rather than cytotoxic effects. In comparison, docetaxel (20 mg/kg Q7D x 4) caused established PC-3 tumors to regress. By day

39, tumors in docetaxel treated animals were still measurable but were 1/4 the size they were when treatment was initiated. On day 39, there was no statistically significant difference in tumor size in mice treated with Rh2 or docetaxel (p=0.179).

It should be noted that docetaxel administration was associated with almost 20% body weight loss (Figure 2.4B, filled squares), and this significant (p=0.025) weight loss compared to saline is consistent with what would be expected for docetaxel administered at its MTD. In comparison, treatment with Rh2 (Figure 2.4B, open triangles) resulted in < 8% loss in mean body weight. This toxicity could be attributed to the repeated animal handling required for daily gavages since the vehicle alone (filled circle) engendered similar, albeit not significant reductions in mean body weight. Interestingly ALT, AST, and ALT/AST levels of Rh2 were significantly lower than the untreated group (p = 0.0001, 0.0149, and 0.0001, respectively) (Table 2.2). There was no statistical difference in creatinine levels between the Rh2 and the control (p = 0.5150) or untreated group (p = 0.3247) (Table 2.2). No statistically significant difference in creatinine level was also found between the control and untreated groups (p = 0.0869). In summary, treatment with Rh2 was achieved at a dose which was well tolerated by the animals.

As other measures of therapeutic activity, PC-3 tumors were isolated from mice at the end of the study and prepared for immunohistochemical assessments of apoptosis and inhibition of Ki-67 labeling. The results, summarized in Figure 2.4D and E, indicate that tumors from mice treated with docetaxel exhibited the highest apoptotic index. The average apoptotic index for the control groups were 9.30 and 7.30% for the oral control and saline respectively. These increased to 14.4% in tumors from animals treated with Rh2 and 22.6% in tumors from animals treated with docetaxel. The apoptotic index observed in mice treated with docetaxel was significantly

greater than the control groups (p=0.022 (oral control) and p=0.015 (saline)) but not significantly different than that observed in tumors from Rh2 treated animals (p = 0.127). The apoptotic index in tumors from animals treated with Rh2 were greater than those observed for controls, but this difference was not significantly different (p=0.079 (oral control and p=0.054 (saline)) (Figure 2.4D). Ki-67 positive cells in tumors from mice treated with Rh2 were lower than that determined for the vehicle control or those tumors derived from docetaxel treated animals. Of the Rh2 tumors, 6.1% were Ki-67 positive whereas the control groups exhibited a significantly higher levels of 23.4 and 21.3% positive staining for the oral control and saline respectively (p=0.003 (oral control) and 0.011 (saline)) and tumors from the docetaxel treated groups exhibited 14.4% (p=0.014 (oral control) and p=0.02 (saline)) positive staining (Figure 2.4E).

2.4 Discussion

We have described a novel oral dosage formulation for ginsenoside Rh2. Pharmacokinetic analysis of Rh2 following oral administration indicates that Rh2 is absorbed when administered by oral gavage. At the highest achievable dose (120 mg/kg) with this formulation, Rh2 proved to be well tolerated in the nude mouse model used and the systemic blood levels achieved following daily gavage were sufficient to prevent progression of established PC-3 tumors. Biodistribution data indicated that intact Rh2 could be isolated from prostate tissue and established PC-3 tumors following oral administration. In aggregate, the results presented here provide strong support for our efforts to develop Rh2 for clinical evaluations and we are particularly interested in the potential of using this agent in combination with docetaxel for treatment of relapsed and hormone insensitive metastatic prostate cancer. The discussion here will therefore consider the potential of Rh2 and its appropriate formulation for use as a well-tolerated drug with potential to augment the therapeutic effects of current chemotherapy.

Rh2 (Figure 2.1A) is an extremely hydrophobic molecule and presents some formulation challenges. Although we are interested in the development of systemic formulations for this ginsenoside, the focus here was to define a suitable formulation for oral administration. The solubility of Rh2 in water miscible solvents such as ethanol is enhanced by the presence of the glucose sugar moiety located at position C-3 on the steroid skeleton. Since the glucose moiety contributes four extra hydroxyl groups, more hydrogen bonds can be established with ethanol. Hydrophobic compounds that lack comparable functional groups typically have a greater preference for solvents with low or zero dipole moments and dielectric constants. Ethanol, however, with a dielectric constant of 24.6 is a better solvent for Rh2 compared to water which possesses a dielectric constant of 80.1 (James, 1986). Ethanol was used to dissolve Rh2 at a

concentration of 130 mg/ml and this solubilized material was then mixed with propylene glycol and water to define an oral dosage formulation. As noted in Figure 2.1B, accelerated stability studies with Rh2 in ethanol at three temperatures suggested that Rh2 could be stored at 4°C or room temperature for at least one month without any significant change in concentration. With a boiling point of 78.15°C for ethanol (O'Neil *et al*, 2001), we also chose to evaluate Rh2 stability at 60°C, maintaining the samples in sealed vials to minimize evaporation. Rh2 was unstable when incubated at this higher temperature. Degradation was observed within 48 hours and progressed rapidly with nearly 80% degradation observed within 14 days.

The final formulation selected for use in oral Rh2 administration comprised ethanol, propylene glycol and water mixed at a ratio (v:v) of 2:7:1. Propylene glycol is extensively used both as a solvent for water-insoluble drugs and as a preservative in topical, parenteral, and oral formulations (Nagatomo *et al*, 2001). Propylene glycol has been shown to be relatively innocuous (LD_{50}=21 g/kg) in acute oral toxicity studies with rats (Lanigan, 2001; Nagatomo *et al*, 2001). Using the ethanol-propylene glycol-water ternary solvent system diagram designed by Sorby *et al.* (Sorby *et al*, 1963), the dielectric constant of the formulated Rh2 oral gavage mixture was estimated to be 31, ideal for the solubility of Rh2.

In addition to defining a suitable formulation for use in *in vivo* studies, it was essential to have appropriate analytical methods in place to help assess drug stability as well as Rh2 pharmacokinetic and biodistribution behavior following administration. Therefore, a significant portion of the work described here concerns development and partial validation of an analytical assay for Rh2. As summarized earlier, the LOD was estimated to be 2.0 ng/ml while the LOQ value was 5.0 ng/ml. Xie *et al.* obtained an LOQ of 2 ng/ml for Rh2 in rat plasma and estimated that plasma concentrations of Rh2 could be reliably quantitated between 2 and 100 ng/ml (Xie *et*

al, 2005b). The mobile phase used by these investigators was supplemented with ammonium chloride. Our LOD and LOQ values in 100% ethanol were comparable to Xie *et al.* We have since shown that the method developed here can reliably be used for the determination of Rh2 in mouse plasma and tissues, including prostate and tumor tissues. The robustness of our method is evidenced by the linearity of the tissue calibration curves with excellent correlation factors of 0.98 or greater (Figure 2.2).

Using the LC/MS assay described above, pharmacokinetic studies were completed after oral administration of Rh2 in the ethanol, propylene glycol and water formulation. The data summarized in Figures 2.3 clearly demonstrates that Rh2 is bioavailable after oral gavage. These results are consistent with a previous study of ginsenoside K, a compound of similar structure to Rh2 which has one glucose moiety at C-20 instead of C-3. Paek *et al.* obtained an AUC of 341.0 (μg.min/ml) following a 20 mg/kg dose of ginsenoside K administered to rats. The Rh2 AUC determined in mice (Table 2.1B) was six times that value, however the dose administered in these studies was six times greater than that used by Paek *et al.* Thirty minutes following administration, < 2% of the administered dose could be found in the stomach, with 54% of the Rh2 administered dose found in the small intestine and 27% of the Rh2 administered dose found in the liver (Figure 2.3A). Considering that Rh2 is being investigated for the treatment of prostate cancer, mouse prostates and tumors were harvested at more frequent intervals, compared to the other tissues. Approximately 0.1% of the administered dose could be isolated from prostate tissue 120 minutes after administration (Figure 2.3E). Similarly, in the subcutaneous PC-3 tumors, approximately 0.3% of the administered dose could be isolated from the tumor 90 minutes after administration (Figure 2.3F). The biodistribution data presented here are consistent with the conclusion that Rh2 administered orally in the ethanol, propylene glycol and water

formulation is readily absorbed and accesses key target tissues in an intact, unmetabolized form. Paek *et al.* determined the transport rate in Caco-2 cell monolayers to be 3-6 x 10^{-6} cm/s for ginsenoside K. Since a transport rate of 2 x 10^{-6} cm/s corresponds to almost complete absorption of an agent in the human intestine, they concluded that ginsenoside K should be well absorbed by the intestine (Paek *et al*, 2006). The biodistribution data summarized in Figure 2.3 is consistent with the ginsenoside K results. In fact, Hasegawa et al. (Hasegawa *et al*, 2000) showed that ginsenoside K also accumulated in the liver following oral administration and they concluded that this accumulation was in part the result of hepatocyte receptors capable of recognizing and binding the associated glucose moiety.

It should be noted that others have shown that Rh2 could be metabolized through deglycosylation to aPPD, its aglycone derivative (Ota *et al*, 1991). Further, Karikura *et al.* showed that ginsenoside hydrolysis and associated cleavage of linked sugar moieties occurs rapidly in 0.1 N HCl, an acidity comparable to that of gastric juice (Karikura *et al*, 1991). It has been suggested that this metabolism was also observed in the intestine, following oral administration of Rh2 (Xie *et al*, 2005a). Importantly, the LC/MS method developed here could also detect aPPD (results not shown) yet there was no evidence of aPPD in the plasma or tissues extracted from Rh2 treated mice. Secondary metabolites of ginsenosides such as Rh2 have also been suspected of being responsible for the observed pharmacological activities. By assessing the primary aglycone metabolite generated by intestinal bacteria, Hasegawa et al. suggested that secondary metabolites may form in the liver. These esterified complexes with fatty acids are believed to be responsible for the tumour growth inhibitory effect generally attributed to ginsenosides and their aglycones (Hasegawa *et al*, 2000). We have demonstrated the presence of intact unmetabolized Rh2 in the blood and every harvested tissue examined, including prostate

and tumor tissue. Future investigation might involve detection and quantitation of possible esterified secondary metabolites from the liver of Rh2 treated mice. As discussed earlier, we did not detect the presence of aPPD, the aglycone of Rh2 in the blood or any tissue. Nonetheless, our group has evaluated an aPPD formulation in a manner comparable to that which is reported here as part of a separate study.

Importantly, the formulation developed here was well tolerated when given (120 mg/kg) QD x 5 each week for four weeks. The mice monitored in our study showed no acute signs of toxicity as determined by body weight loss (Figure 2.4B) and this observation is consistent with other studies (Chen *et al*, 1980) that suggest that ginsenosides in general, and Rh2 in particular, cause negligible toxicity, even at the maximal achievable dose. As shown in Table 2.2, secondary measures of liver toxicity also suggest the drug is well tolerated. Serum ALT and AST activity following Rh2 administration were not increased. ALT and AST values for all three groups were within the expected range for normal mice (ALT $\leq$ 50 U/l and AST $\leq$ 150 U/l) (Saad *et al*, 1993). Serum creatinine levels were determined as a measure of kidney toxicity and the results suggest that there was no statistical difference in creatinine levels between the Rh2 and the control ($p = 0.5150$) or untreated group ($p = 0.3247$) (Table 2.2). The untreated group was also not statistically different from the control group ($p = 0.0869$). Despite the apparent difference in serum creatinine level between the untreated and control group, values for all three groups were within the expected range for normal mice (0.05 to 0.15 mg/dl) (Dunn *et al*, 2004), suggesting no significant toxic effects of Rh2 on this organ.

Consistent with previous findings (Nakata *et al*, 1998), Rh2 appears to exhibit therapeutic activity when used to treat established tumors derived following subcutaneous injection of PC-3 cells (Figure 2.4A) and the results suggest that the activity, albeit lower, was comparable to that

achieved when treating mice with docetaxel administered at its MTD. Nakata *et al.* demonstrated that a daily oral administration of Rh2 to nude mice bearing HRA human ovarian cancer xenograft caused an inhibitory effect comparable to a weekly administration of 4 mg/kg cisplatin (Nakata *et al*, 1998). Like most ginsenosides, Rh2 treated mice showed no signs of toxicity while nearly half of cisplatin-treated mice had to be terminated due to acute body weight loss. Interestingly, Nakata *et al* attributed the therapeutic effects of Rh2 to enhanced NK activity however our work in immunocompromised mice suggests additional mechanisms may also be involved (Nakata *et al*, 1998). To better understand the nature of Rh2 mediated therapeutic effects, PC-3 tumors were isolated at the end of the treatment phase of the study and measures of apoptosis (TUNEL staining) and proliferation (Ki-67) were made. The apoptotic index of the docetaxel group was significantly higher than the control groups (p=0.022 (oral control) and p=0.015 (saline)). While the value determined in tumors from animals treated with the Rh2 group were approximately 50% greater than that seen for control tumors, this difference was not statistically significantly (p=0.079 (oral control and p=0.054 (saline)) (Figure 2.4D). Others have shown that ginsenosides are strong promoters of apoptosis in a rat glioma model (Kim *et al*, 1999), a human melanoma model (Fei *et al*, 2002), an ovarian cancer model (Nakata *et al*, 1998), a breast cancer model (Oh *et al*, 1999), a hepatocellular carcinoma model (Park *et al*, 1997) as well as in models of neuroblastoma (Kim *et al*, 2000). The fact that we did not observe significant increases in the apoptotic index may be related to the time points used in our study. In our study we demonstrated that Rh2 significantly inhibited cell proliferation as measured by Ki-67 labeling (Figure 2.4E) and the extent of suppression was significantly lower than what was observed in tumors isolated from animals treated with docetaxel.

In summary, the formulation that we describe here is well tolerated and effective when used to treat established PC-3 tumors. Pharmacokinetic and biodistribution studies suggest that Rh2 is well absorbed. Future studies will assess the activity of this ginsenoside when used in combination with conventional cytotoxic/cytostatic drugs, in particular docetaxel, *in vitro* and *in vivo*. The efficacy results shown here indicate that continuous dosing of Rh2 will mediate effects that suppress tumor growth (i.e. results in stable disease), but it is understood that tumor growth will recur once treatment has stopped. This cytostatic effect may be important when using Rh2 in a combination setting with a chemotherapeutic known to cause regression of established tumors, as was observed here when PC-3 tumor bearing mice were treated with docetaxel.

2.5 Acknowledgements

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2.7 Tables

Table 2.1. **(A) Average extraction efficiencies of Rh2 in plasma and tissues. Extraction standards were completed using three concentrations (0.5, 2.0, and 5.0 μg/ml) of Rh2 and each assay was determined in triplicate. (B) Pharmacokinetic parameters for Rh2 following oral administration of 120 mg/kg formulated in the ethanol-propylene glycol-water ternary solvent system described in the Methods. Since n = 3 per time point, three values were obtained for each parameter listed in the table. Values are reported as mean ± SEM.**

A

Plasma (%)	Stomach (%)	Small intestine (%)	Liver (%)	Lung (%)	Spleen (%)	Kidney (%)	Brain (%)	Prostate (%)	Tumour (%)
65.2±2.4	79.6±3.2	99.0±10.7	92.9±2.6	90.3±10.0	96.5±2.7	111.5±7.1	108.0±5.9	86.2±3.1	93.4±3.7

B

Body Weight (g)	$t_{1/2}$ (min)	C_{max} (μg/ml)	Cl_{oral} (ml/min/kg)	AUC (μg.min/ml)
25.8 ± 1.3	99.6 ± 6.6	19.0 ± 2.0	46.5 ± 2.7	1884.3 ± 209.2

$t_{1/2}$: Half-life
C_{max}: Peak Concentration
Cl_{oral}: Apparent oral clearance
AUC: Area under the plasma concentration versus time curve

Table 2.2. Secondary measures of toxicity in mice serum (n = 4). Values are reported as mean ± SEM.

Group	ALT (Alanine aminotransferase) (U/L)	AST (Aspartate aminotransferase) (U/L)	ALT/AST	Creatinine (μg/ml)
Rh2	19.57 ± 0.2*	48.39 ± 0.4**	0.36 ± 0.003*	1.11 ± 0.14***
Oral control	23.09 ± 0.2	54.60 ± 0.3	0.48 ± 0.005	1.21 ± 0.07
Untreated	24.63 ± 0.2	52.06 ± 0.6	0.47 ± 0.006	0.84 ± 0.22

P values – Rh2 compared with the untreated group
* $p = 0.0001$
** $p = 0.0149$
*** $p = 0.3247$
U/L: Units per litre

2.8 Figure legends

Figure Legends

Figure 2.1. (a) Chemical structure of ginsenoside Rh2, a 20(S)-protopanaxadiol dammarane skeleton aglycone type. (b) Stability of Rh2 in 100% ethanol for 28 days. Conditions are 4°C (empty circles), room temperature (R.T., filled squares), and 60°C (filled triangles), n = 3. (c) Mean plasma concentration-time profile of Rh2 in mice following oral administration of 120 mg/kg, n = 3 per time point.

Figure 2.2. Tissue calibration curves determined using HPLC for Rh2 added to homogenates prepared from (a) Stomach, (b) Small intestine, (c) Liver, (d) Kidney, (e) Spleen, (f) Lung, (g) Brain. r^2 values are ≥ 0.98, n = 3. a.u.c.: area under the curve obtained from the Rh2 peak eluting from the HPLC. Error bars represent standard Error of the means.

Figure 2.3. (a)-(d): % administered dose of Rh2 in specified tissues isolated from mice following oral administration of 120 mg/kg. (a) 30 minutes (b) 60 minutes (c) 120 minutes (d) 200 minutes. E-F: % administered dose of Rh2 in the prostate (e) and tumors (f) isolated from mice 10, 20, 30, 45, 60, 90, 120, 150, and 200 minutes following oral administration of 120 mg/kg. Data was obtained from three animals per time point and results are expressed as mean values ± SEM.

Figure 2.4. (a) *In vivo* efficacy of Rh2 in PC-3 bearing nude mice. Changes in tumor volume were followed over time for animals treated with the oral gavage vehicle control (ethanol, propylene glycol and water, filled circle), saline (x), Rh2 (120 mg/kg QD x 5, empty triangle), and docetaxel (20mg/kg Q7D x 4, filled square). Average tumor volumes are expressed as a percentage of the average initial tumor volume of each group at day 14, post PC-3 cells inoculation. Volumes were calculated using $V = (LxWxH)/(\pi/6)$. Mean value ± SEM is shown with error bars. A statistically significant difference was found between Rh2 and the control (p=0.0122) and saline (p=0.0081) on

day 39. No statistical difference between Rh2 and docetaxel (p=0.1787). n = 8 for Rh2 and docetaxel. n = 6 for the oral gavage control and saline. (b) *In vivo* toxicity as assessed by decreases in mean body weight. Body weight loss of > 20% is considered severe enough to warrant termination of the animals. No animals showed any signs of toxicity other then weight loss in these studies. Docetaxel showed a significant weight loss compared to saline (p=0.0246). n = 8 for Rh2 and docetaxel. n = 6 for the oral gavage control and saline. (c) Representative tissue microarray spots for PC-3 tumors: immunostained using a TUNEL assay by enzymatically labeling the free 3'-OH terminal generated on DNA fragments of 180 to 200 bp. Using this method, apoptotic cells could be identified in areas of viable tissue, as indicated by the arrows. (d) Apoptotic Index summarized from ApopTag® staining of tissue microarrays. (e) summarized Ki-67 staining of tissue microarrays. Mean scores were determined as a percentage of the total number of cells. Four cores of tissue were extracted per tumor and one tumor per animal. n (total number of cores) = 24 for the control and saline and 32 for Rh2 and docetaxel. Mean value ± SEM is shown with error bars. * signifies statistically significant difference from the control.

2.9 Figures

Figure 2.1

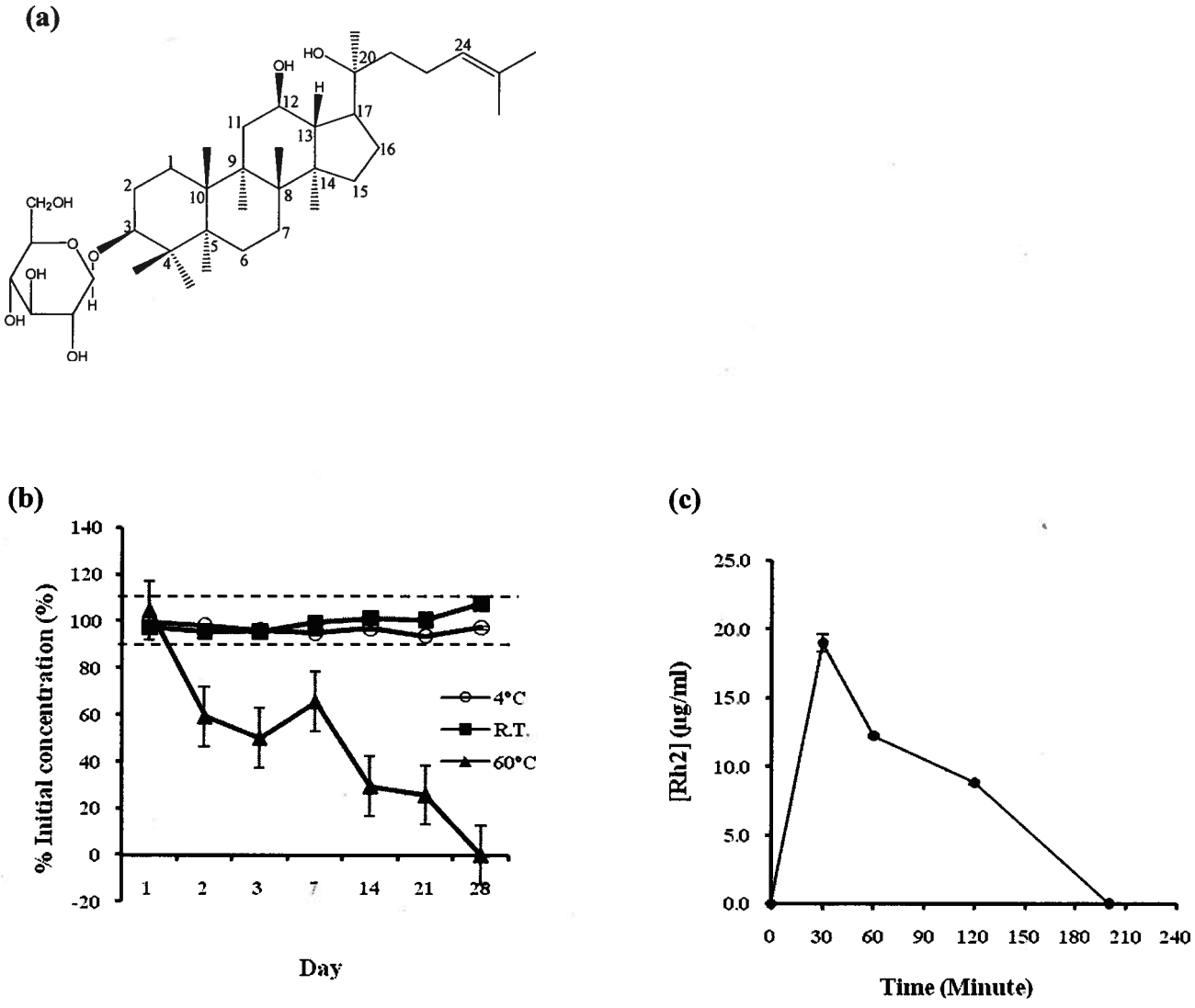


Figure 2.2

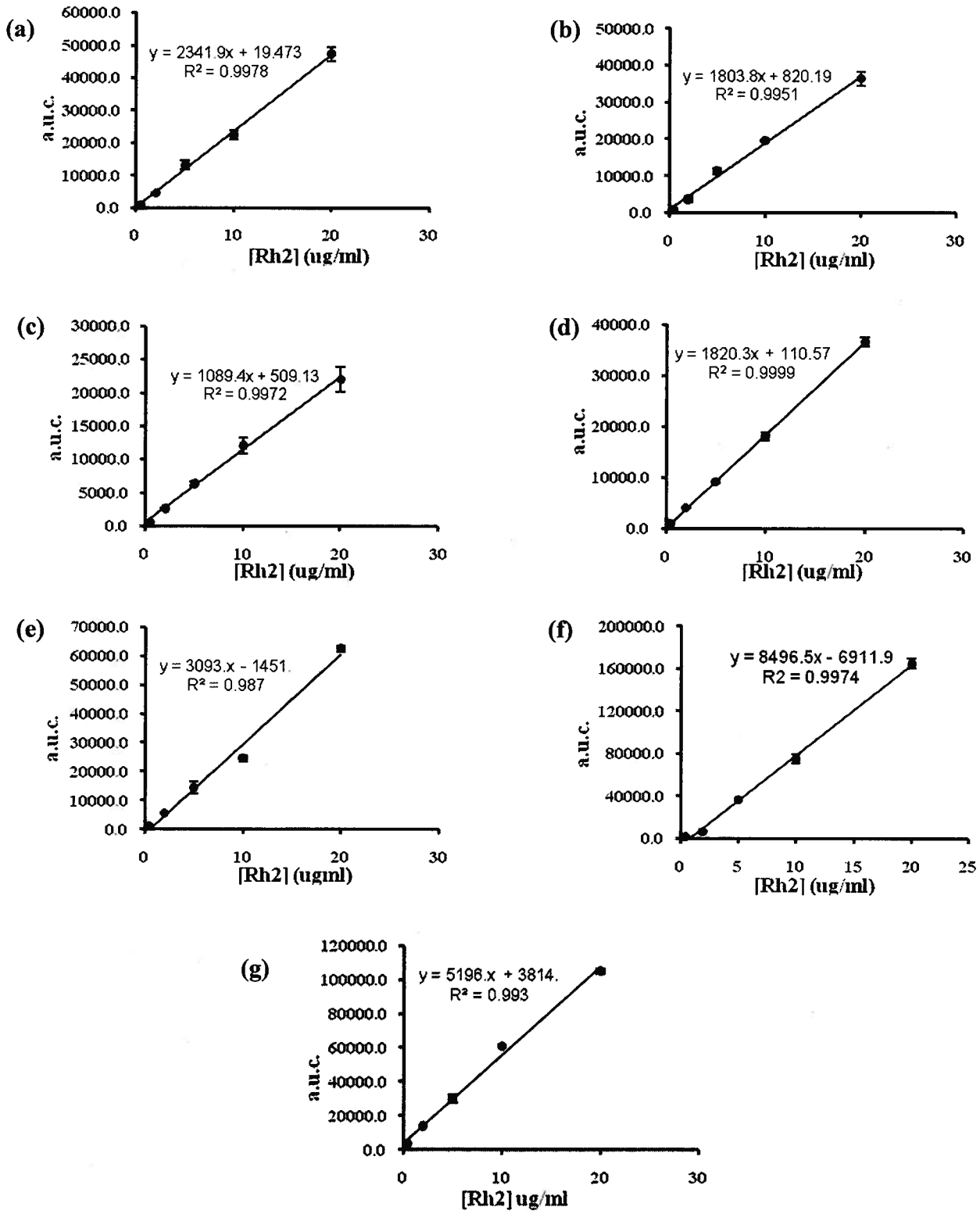

(a)
y = 2341.9x + 19.473
R² = 0.9978
a.u.c.
[Rh2] (ug/ml)
(b)
y = 1803.8x + 820.19
R² = 0.9951
a.u.c.
[Rh2] (ug/ml)
(c)
y = 1089.4x + 509.13
R² = 0.9972
a.u.c.
[Rh2] (ug/ml)
(d)
y = 1820.3x + 110.57
R² = 0.9999
a.u.c.
[Rh2] (ug/ml)
(e)
y = 3093.x - 1451.
R² = 0.987
a.u.c.
[Rh2] (ugml)
(f)
y = 8496.5x - 6911.9
R2 = 0.9974
a.u.c.
[Rh2] (ug/ml)
(g)
y = 5196.x + 3814.
R² = 0.993
a.u.c.
[Rh2] ug/ml

Figure 2.3

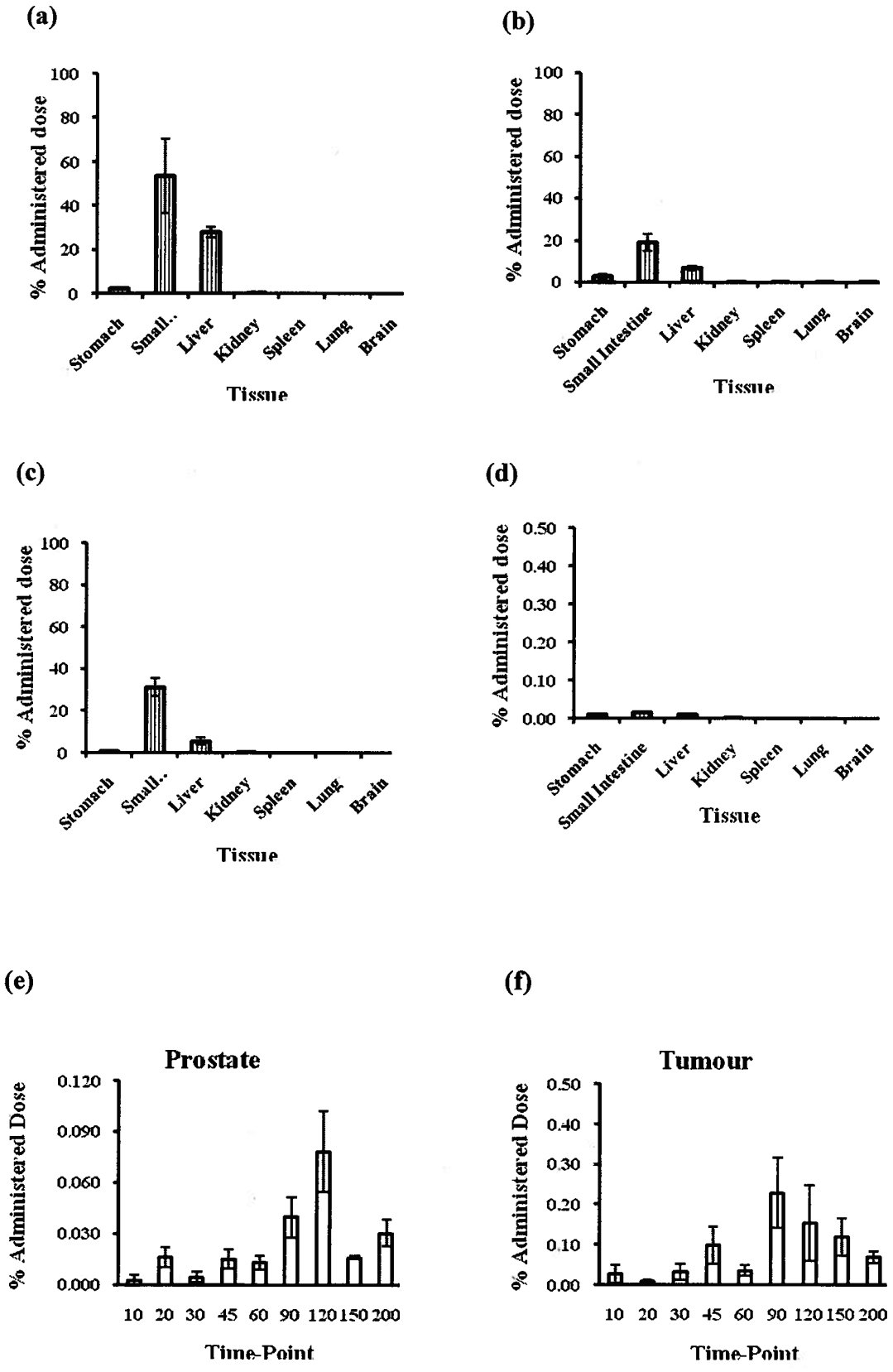
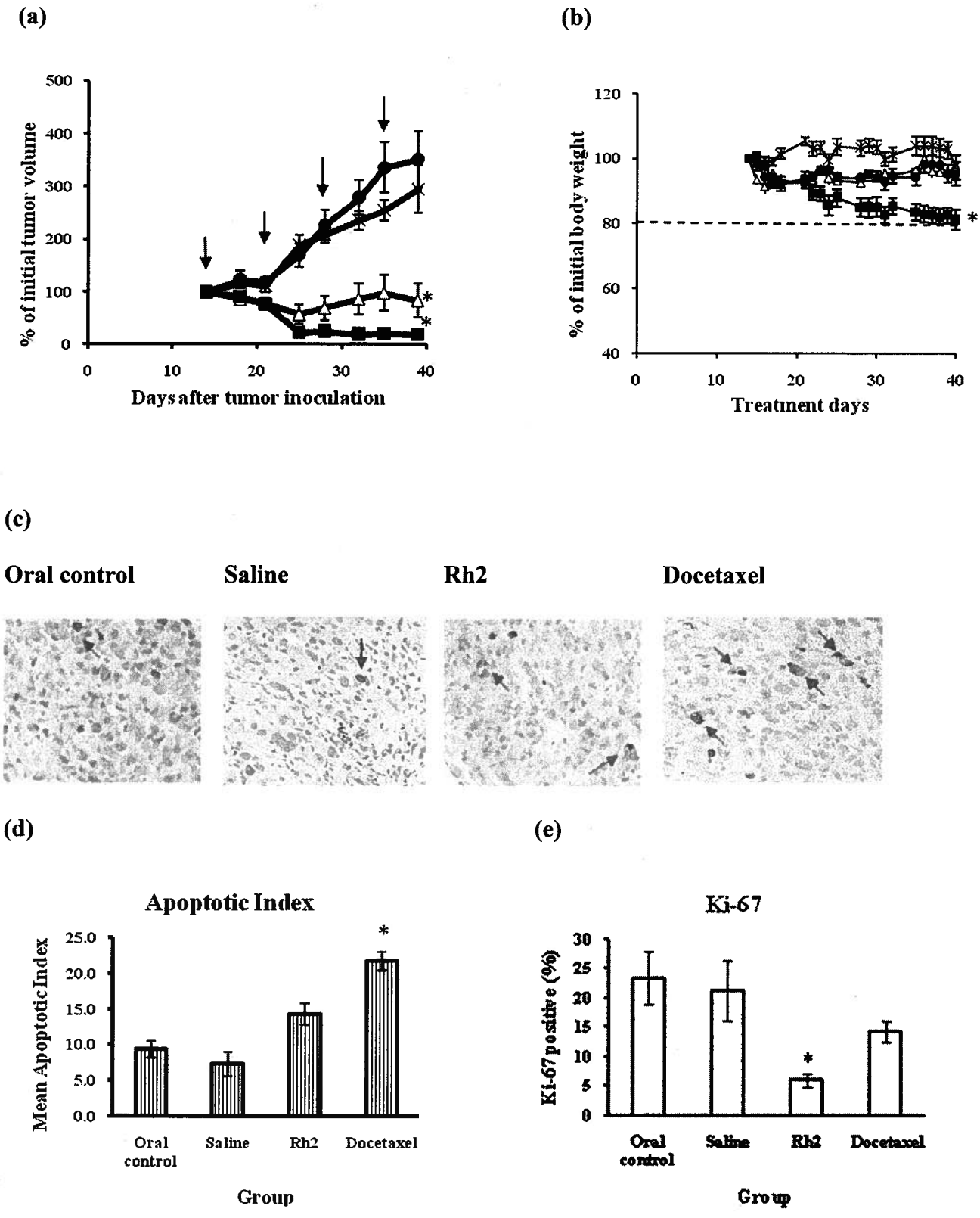
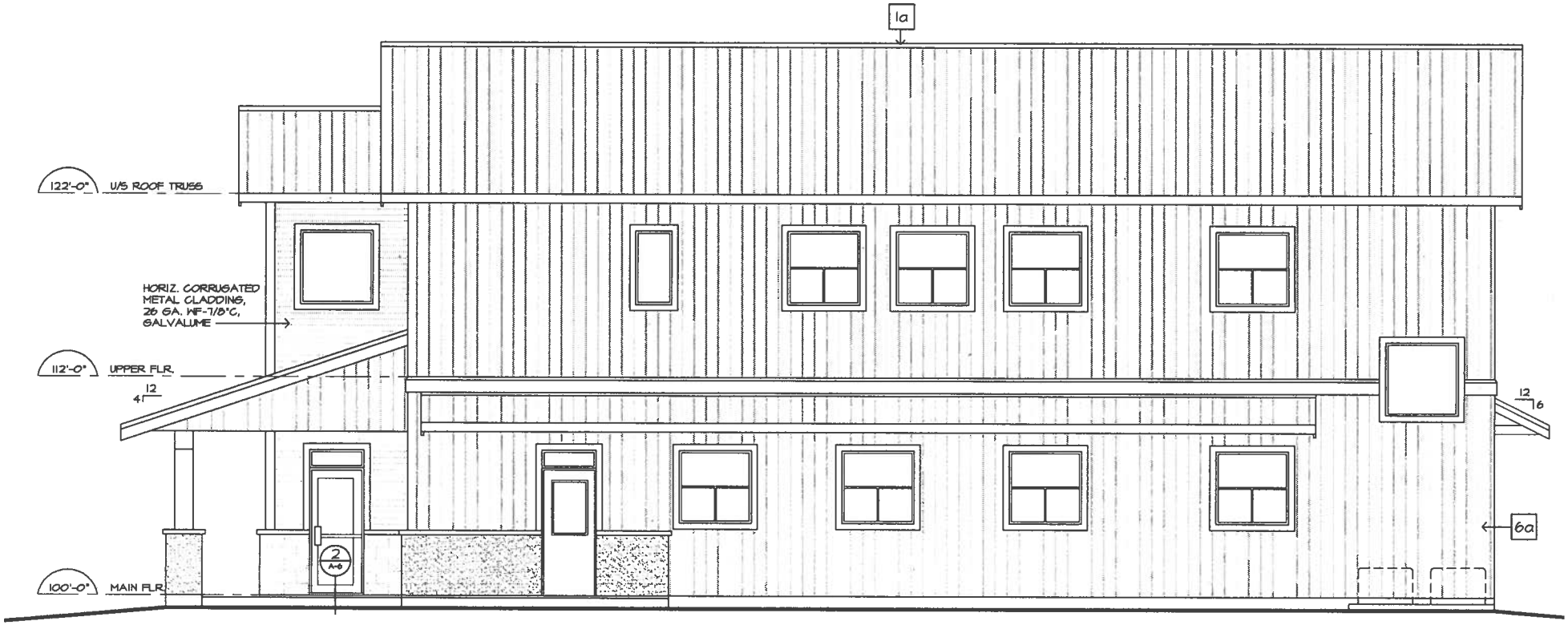

(a)
% Administered dose
100
80
60
40
20
0
Stomach
Small..
Liver
Kidney
Spleen
Lung
Brain
Tissue
(b)
% Administered dose
100
80
60
40
20
0
Stomach
Small Intestine
Liver
Kidney
Spleen
Lung
Brain
Tissue
(c)
% Administered dose
100
80
60
40
20
0
Stomach
Small..
Liver
Kidney
Spleen
Lung
Brain
Tissue
(d)
% Administered dose
0.50
0.40
0.30
0.20
0.10
0.00
Stomach
Small Intestine
Liver
Kidney
Spleen
Lung
Brain
Tissue
(e)
Prostate
% Administered Dose
0.120
0.090
0.060
0.030
0.000
10 20 30 45 60 90 120 150 200
Time-Point
(f)
Tumour
% Administered Dose
0.50
0.40
0.30
0.20
0.10
0.00
10 20 30 45 60 90 120 150 200
Time-Point

Figure 2.4


(a)
% of initial tumor volume
Days after tumor inoculation
(b)
% of initial body weight
Treatment days
(c)
Oral control
Saline
Rh2
Docetaxel
(d)
Apoptotic Index
Mean Apoptotic Index
Oral control
Saline
Rh2
Docetaxel
Group
(e)
Ki-67
Ki-67 positive (%)
Oral control
Saline
Rh2
Docetaxel
Group



SOUTH ELEVATION

SCALE: 1/4"=1'-0"

3. SECOND MANUSCRIPT CHAPTER

A novel oral dosage formulation of the ginsenoside aglycone aPPD exhibits therapeutic activity against a hormone insensitive model of prostate cancer[1]

[1] A version of this chapter has been submitted for publication. Co-authors: Andy Eberding, Catherine Wood, Hans Adomat, Ladan Fazli, Antonio Hurtado-Coll, William Jia, Marcel B. Bally, and Emma Tomlinson Guns.

3.1 Introduction

It is known that cancer patients often take complementary and alternative medicines (CAMs) while being treated with the best available treatment options (Richardson *et al*, 2000). Most patients consider use of CAMs because they feel the product will not do harm, based on an assumption of lack of toxicity (Richardson *et al*, 2000), while also providing potential therapeutic benefits. As one example, ginseng (Panax ginseng C.A. Meyer) has been used for medicinal purposes for a long time and proponents for its use claim that ginseng extracts are effective in treating a wide range of disorders including cancer (Duda *et al*, 1999; Nah *et al*, 1995; Yun *et al*, 2001; Zhang *et al*, 1996). Although the use of ginseng extracts for treatment of prostate cancer can be questioned, it is now well established that there are many active compounds within a given ginseng extract that exhibit measurable anticancer effects (Fei *et al*, 2002; Kim *et al*, 1999; Kim *et al*, 2000; Oh *et al*, 1999; Park *et al*, 1997). It is not unreasonable to pursue the development and use of these active components for treatment of cancer and our research team has an interest in developing selective ginsenosides for use in treatment of advanced prostate cancer.

From a nutraceutical perspective, this approach has been criticized in part because it can be argued that the therapeutic benefits arising from ginseng extracts are a result of the neutraceutical "cocktail" that exists in the extract. It is suggested that the combination of active ingredients act synergistically to produce therapeutic effects greater than that which can be achieved with the single active components (Budman *et al*, 2002; Chou & Talalay, 1984; Frei & Antman, 1997; Xie *et al*, 2006). It is obviously difficult to address this without first establishing the activities of individual components and their metabolites. This work is complex and arduous, even if one assumed that all active components could be identified and isolated. Our approach has been to consider the development of isolated ginsenosides in combination with medicines

that are part of existing standards of care (Xie *et al*, 2006). Here again, it is critical to first define the therapeutic effects achieved when the identified ginsenoside is used alone, prior to considering its use in combination with other chemotherapy drugs.

Our laboratory has recently reported on the anti-prostate cancer activity of the ginsenoside Rh2 (manuscript submitted). Rh2 is a tri-terpenoid glycoside saponin found in trace quantities in extracts from the roots of *Panax ginseng* C.A. Meyer. However, it is one of the major *in vivo* metabolic products of ginseng when taken orally (Jin *et al*, 2006; Popovich & Kitts, 2004). Others have demonstrated that Rh2 can be degraded, through a deglycosylation reaction, to aPPD (Figure 3.1A) and it has been suggested that this degradation occurs in the intestine following oral administration of Rh2 (Bae *et al*, 2004). Although we anticipated that treatment with Rh2 would result in systemic exposure to both Rh2 and aPPD, the LC/MS methods used to assess the plasma concentration of these agents indicated that there was no detectable level of aPPD in the plasma or tissues extracted from Rh2 treated mice (manuscript submitted). Further, since aPPD has been reported to exert its own therapeutic effects (including: (i) inducing cell cycle arrest in G1(Popovich & Kitts, 2002); (ii) inducing apoptosis (Wang *et al*, 2008b) and/or autophagy (Liu *et al*, 2007); and (iii) exhibiting anti-estrogen activity (Yu *et al*, 2007)), it is important to characterize its effect when used as a single agent *in vivo*. The studies described here evaluate the pharmacokinetic and biodistribution behavior of aPPD formulated for oral administration. In addition, a preliminary assessment of treatment induced toxicities and therapeutic activity was completed. Overall, the research described here is significant and relevant to the development of aPPD as a therapeutic agent for castrate resistant prostate cancer.

3.2 Materials and methods

Test compounds and materials: aPPD was purchased from LKT Laboratories Inc., St. Paul, MN. (USA) or generously supplied by Panagin Pharmaceuticals Inc, Richmond, BC (Canada). Taxotere® (docetaxel) was manufactured by Aventis Pharma Inc. Saint-Laurent, Québec (Canada). Cholic acid and all other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

LC/MS analysis of aPPD:

The HPLC system consisted of an integrated Waters Alliance 2695 quarternary solvent delivery system with low pressure mixing, plus autosampler and 996 photodiode array detector (Waters Corp., Milford, MA). aPPD and cholic acid (internal standard) were resolved on an Exterra C18 column (3.5 μm, 50 mm x 2.1 mm; Waters, Milford, Massachussets) with Mobile Phases ddH_2O, methanol, acetonitrile, and 0.5% acetic acid. A Waters/Micromass ZQ 2000 detector and MassLynx version 3.4 (Micromass UK Ltd, Manchester, UK) were also used. Standard solutions of aPPD were prepared between 0.01 and 500.0 μg/ml (n=3). The Limit of Detection (LOD), or the minimum concentration that could be detected based on a signal-to-noise (S/N) ratio of 3 to 1, was defined. The Limit of Quantification (LOQ) was also determined. The LOQ was the minimum concentration that could be quantified based on S/N ratio of 10 to 1. The estimated LOD and LOQ were determined to be 5.0 ng/ml and 20.0 ng/ml, respectively.

In order to define the solubility of aPPD in ethanol, three sets of saturated solutions of aPPD were prepared at room temperature by incubating an excess of the compound in 2 ml of 100% ethanol. The mixture was mixed and stored at room temperature. After 30 minutes, the material that was not dissolved precipitated in the tube. The amount of aPPD in the clear supernatant was quantified by HPLC. These data indicated that the maximum solubility of aPPD was 68.4 ± 10.0 mg/ml. Subsequently, stability studies were completed by first preparing a

solution of 120 mg of aPPD dissolved in 1.8 ml of 100% ethanol. This solution was pipetted (200 µL) into each of nine eppendorf tubes. One set of three tubes was stored at 4°C, room temperature, and 60°C. The concentration of aPPD in these solutions was determined by HPLC on day 1, 2, 3, 7, 14, 21 and 28. This study indicated that aPPD was stable (<5% loss of parent drug) in ethanol at 4°C and room temperature for time frames of at least 1 month. aPPD underwent rapid thermal degradation, measured as a loss of intact drug, at 60°C (Figure 3.1B). A concentration change of ± 15% is generally considered acceptable(USP, 2000).

For analysis of aPPD in plasma and tissues; aPPD extraction efficiencies were first assessed by spiking blank plasma and tissue homogenate samples with aPPD and cholic acid to achieve three concentrations (0.5, 2.0, and 10.0 µg/ml). For plasma samples, the mixtures were placed on C18 Sep-Pak extraction columns mounted on a vacuum extraction manifold (Waters, Massachusetts, USA). The C18 Sep-Pak columns were first equilibrated with 1 ml of 100% methanol and 1 ml of ddH_2O. After loading with 200 µl of plasma spiked with 10 µl of cholic acid, each column was subsequently washed with 1 ml of ddH_2O followed by 1 ml of 30% methanol. The column was then eluted twice with 1 ml of 100% methanol, the eluate was dried, the residue reconstituted with 100 µl of 100% methanol and transferred into HPLC. Tissue samples were ultrasonicated in 4 x w/v of 0.01 M pH 7.8 phosphate buffer. 200 µl of the homogenate was spiked with cholic acid and vortexed. Ice cold acetonitrile (800 µl) was added, vortexed, and the sample centrifuged at 12,000 g for 15 minutes. The supernatant was dried, the residue reconstituted in 200 µl of 100% methanol and injected into the HPLC column. Plasma or tissue standard calibration curves (results not shown), exhibited excellent linearity (correlation coefficients of 0.97 or greater). The extraction efficiency from plasma was 59.0 ± 2.4% and this was not influenced by the concentration of aPPD used in these assays (Table 3.1A). Extraction

efficiencies from tissues vary from 77.5 ± 4.7% in stomach to essentially 100% in the brain (Table 3.1A).

Oral gavage formulation:

We recently described a formulation methodology suitable for Rh2 (manuscript submitted) and this method was also used for aPPD. In brief, 120 mg of aPPD was dissolved and mixed in 1.8 ml of 100% ethanol (based on maximum solubility of 68.4 mg/ml). Subsequently 700 µl of ddH_2O and 5.5 ml of propylene glycol were added and mixed. The resulting clear solution was then used for oral administration to mice. The highest daily dose of aPPD achievable using this formulation was 70 mg/kg assuming a maximum gavage volume of no more than 150 µL.

***In vivo* studies:**

For pharmacokinetic and biodistribution studies five groups of three mice were dosed by oral gavage with aPPD (70 mg/kg) or the vehicle control (117-145 µl). Mice were terminated (by CO_2 asphyxiation) at 30, 60, 120, and 200 minutes following administration and blood was collected immediately by cardiac puncture and transferred into lithium heparin-containing microtainers. Major organs (stomach, small intestine, liver, kidney, spleen, brain, and lung) were also collected for analysis of aPPD levels. A non-compartmental method using the WinNonlin program (Scientific Consulting Inc., Cary, N.C., USA) was used to generate pharmacokinetic parameters.

For efficacy studies, PC-3 cells (1.0×10^6) were inoculated subcutaneously into 6-8 week old male nude mice (Harlan Sprague Dawley, Inc.) weighing 25-31 g. After 14 days, tumors were measured and mice were then randomly assigned in different treatment groups. A caliper was used to measure each tumor and the formula $V = (L \times W \times H)\frac{\pi}{6}$ where L is the length, W the width, and H the height, was used to calculate the tumor volume. The average tumor volume when treatment was initiated was 120 mm^3. Four different treatment groups were defined: aPPD

(70 mg/kg, p.o. QD x 5 schedule each week for 4 weeks), vehicle control (p.o. QD x 5 schedule each week for 4 weeks), docetaxel (positive control, 20 mg/kg, i.v. Q7D x 4 dose schedule) and saline (i.v. Q7D x 4 dose schedule). Mice were weighed five days a week and tumors measured twice a week. For the aPPD and docetaxel groups the n number of mice per group was defined at 8 while an n = 6 was used for the oral gavage and saline control groups. The mice were monitored daily for signs of acute toxicity.

Analysis of apoptosis and proliferation markers:

At the end of the efficacy studies mice were sacrificed and tumors excised, formalin-fixed, and then paraffin-embedded. Apoptotic cells were visualized by TUNEL using the ApopTag® Peroxidase Kit. Ki-67 (Dako, Carpinteria, California) (1:200) monoclonal antibodies were used in combination with immunoperoxidase procedures (LSAB + peroxidase kit). The TMA slides were imaged digitally and evaluated by visual scoring of apoptotic and Ki-67 positive cells. Scoring was completed by two pathologists blinded to the study groups.

Statistical analysis:

For each studied variable, mean and standard error of the mean (S.E.M.) were calculated. Statistical significance was assessed using the Tukey-Kramer/Student's t-tests from within One-way Analysis of Variance (ANOVA) analysis. The level of significance was set at a p value of at least < 0.05. All mean values were reported as mean ± S.E.M.

3.3 Results

aPPD Pharmacokinetics and Biodistribution:

Drug levels in the plasma peaked at 3.9 ± 1.4 μg/ml, following oral administration of aPPD formulated in the ethanol/water/ propylene glycol mixture (see Methods). After 200 minutes the drug levels were less than the assay's LOQ (20.0 ng/ml) (Figure 3.1C). The biodistribution studies summarized in Figure 3.2, indicated that aPPD levels were highest in the stomach and small intestine 30 minutes after the gavage was administered, with approximately 44 and 32% of the drug recovered in these tissues, respectively (Figure 3.2A). This would be consistent with the route of administration used. Approximately 5% of the administered dose is found in the liver at the same time point (Figure 3.2A). Interestingly, aPPD level was quantifiable in the brain with 0.01% of the initial dose recovered from 30 to 120 minutes after administration (Figure 3.3E).

***In vivo* efficacy and toxicity:**

Mice bearing established PC3 tumors exhibited an average tumor volume of approximately 100 to 140 mm^3, 14 days after tumor cell inoculation. When treated with saline (Q7D x 4) or vehicle control (QD x5 for 4 weeks) these tumors continued to grow until by day 30 the tumor size had increased approximately 3 fold relative to when treatment was started (Figure 3.3A). When treated with docetaxel (20 mg/kg Q7D x 4) the established tumors regressed to ¼ of their size when treatment started. This dose of docetaxel was equivalent to its MTD; docetaxel administration was associated with almost 20% body weight loss (Figure 3.3B, filled squares) and this was significant weight loss (p=0.0001) when compared to saline treated mice. In comparison to this positive control, aPPD given daily for 21 days was less active however the administered dose was effective. The tumor volume increased only by 52% for the aPPD treated animals, which was statistically different from the vehicle (p=0.0044) and saline (p=0.0159)

controls. Treatment with aPPD was associated with < 5% weight loss and was tolerated significantly better then the docetaxel treatment (Figure 3.3B, filled triangles).

As another measure of therapeutic activity, tumors were harvested at day 40 (after cell inoculation, 28 days after treatment was initiated) and assessed immunohistochemically for apoptosis and proliferation (Figure 3.3C-E). The average apoptotic indices were 9.30%, 7.30%, 15.6% and 22.6% for the vehicle control, saline, aPPD, and docetaxel treated groups, respectively. The apoptotic index observed in mice treated with docetaxel was significantly greater than the control groups (p=0.079 (oral control and p=0.054 (saline)) but not significantly different from aPPD treated animals (p = 0.2305). The apoptotic index determined for tumors obtained from aPPD treated mice was not significantly different from tumors obtained from control animals (p=0.0716 (oral control) and p=0.059(saline)) (Figure 3.3D). Ki-67 positive staining (3.2%) in tumor tissues harvested from aPPD treated animals was significantly lower (p=0.0107 (oral control) and p=0.025 (saline)) then that obtained for control tumors (23.4 and 21.3% for the oral control and saline respectively) (Figure 3.3E). Ki-67 positive staining in the aPPD group was also significantly lower (p=0.007) than that observed for tumors from docetaxel treated mice (14.4%) (Figure 3.3E).

3.4 Discussion:

Ginsenoside aglycone aPPD is a stable compound that can be formulated for oral administration using methods that are comparable to those for Rh2 (manuscript submitted). Using the ethanol-propylene glycol-water ternary solvent system diagram designed by Sorby *et al.* (Sorby *et al*, 1963), the dielectric constant of the formulated aPPD oral gavage mixture was estimated to be 31, ideal for the solubility of aPPD. aPPD in ethanol could be stored at 4°C or room temperature for at least one month without any significant change in concentration. Pharmacokinetic studies indicate that aPPD is absorbed when administered in this formulation and the systemic blood levels achieved are sufficient to provide effective treatment of mice bearing established PC-3 tumors. At the highest achievable dose (70 mg/kg based on a maximum gavage volume of 100 to 150 µl), aPPD did not exhibit significant toxicity in the nude mouse model. The results suggest that aPPD should be considered for further development in the treatment of castrate-insensitive prostate cancer. This discussion considers how ginseng metabolites such as aPPD and Rh2 could be used in a combination therapy regimen to treat patients with advanced metastatic prostate cancer.

aPPD is approximately half as soluble as Rh2 in 100 % ethanol and is retained in the gastro-intestinal (GI) tract at significant levels for at least 2 hours following administration (Figure 3.2A-C), most probably because of its higher hydrophobicity. Only 5% of the administered dose reached the liver thirty minutes following administration (Figure 3.2A) compared to 28% for Rh2 (manuscript submitted). Since the pH in mouse small intestine varies between 6.5 and 7.1 (Kararli, 1995), it can also be suggested that aPPD is poorly absorbed because in vitro studies with Caco-2 cells demonstrate it is optimally transported at a pH of 8.0 (Xie *et al*, 2005). Interestingly, the results suggest that aPPD can cross the blood-brain barrier, whereas previous studies with Rh2 indicated that no detectable levels of Rh2 could be found in

the brain following oral administration. It is established that lipophilic drugs penetrate the endothelial cells of the blood-brain barrier more easily than hydrophilic drugs (Rapoport *et al*, 1979) and passive diffusion permeation rates are correlated to lipophilicity (Levin, 1980). In addition, it can be speculated that the accumulation of aPPD in the brain is the result of its effectiveness in stimulating BCRP (Breast Cancer Resistance Protein)-ATPase activity, following BCRP inhibition, which would allow it to be transported by BCRP across the blood-brain barrier (Jin *et al*, 2006).

Mice treated QDx5 for four weeks at an aPPD dose of 70 mg/kg showed no acute signs of toxicity as determined by body weight loss (Figure 3.3B) and this observation is consistent with other studies (Chen *et al*, 1980) that suggest that ginsenosides and their aglycones cause negligible toxicity, even at maximal achievable doses. Since mice treated with Rh2 also do not show acute signs of toxicity, it can be suggested that aPPD and Rh2, both members of the 20 (S)-protopanaxadiol ginsenoside category, share a similar toxicity profile. Secondary measures of liver and kidney toxicity following Rh2 administration in nude mice showed no increase in serum ALT and AST activity and serum creatinine levels were not statistically different from the control group (manuscript submitted). It would be anticipated that similar results would be obtained when using aPPD.

Therapeutic activity of aPPD against PC-3 tumors (Figure 3.3A) is less than that which can be achieved with docetaxel, which was used as a positive control. However, aPPD was well tolerated at the dose used while docetaxel was used at its MTD; causing almost 20% body weight loss in treated animals. The apoptotic index of the aPPD group was not statistically different from the control groups and this was unexpected. Others have shown *in vitro* that aPPD and other ginsenosides are strong promoters of apoptosis (Liu *et al*, 2007; Popovich & Kitts, 2002; Wang *et al*, 2008a). The fact that we did not observe significant increases in the apoptotic index

may be related to the time point used in our study or to the fact that the drug levels achieved within the plasma following oral administration were much lower than that which were used in the *in vitro* studies. However, aPPD significantly inhibited cell proliferation as measured by Ki-67 labeling and this may be attributed to aPPD's ability to engender G1-arrest at low concentration (Popovich & Kitts, 2002).

In conclusion, the aPPD formulation described here is well tolerated and effective when used to treat established PC-3 tumors. Pharmacokinetic and biodistribution studies suggest that aPPD is well absorbed. Future studies will assess the activity of this ginsenoside when used in combination with the drug docetaxel. Despite continual research efforts, few chemotherapeutic drugs have been shown to be effective for prostate cancer treatment in patients who have relapsed and/or have metastatic disease. In recent years, however, drug combinations, and particularly those involving taxanes, have substantially contributed to improvements in treatment outcomes (De Wit, 2008; Oh, 2003) in this patient group. Thus it is reasonable to consider whether appropriately selected and characterized ginsenosides will enhance treatment outcomes achieved when using taxanes.

3.5 Acknowledgements:

The authors wish to thank Panagin Pharmaceuticals Inc and the Staff of the Jack Bell animal resource centre.

Abbreviations and Acronyms

CAM	=	Complementary and Alternative Medicine
MX	=	Mitoxantrone
BCRP	=	Breast Cancer Resistance Protein
S/N	=	Signal-to-Noise ratio
HPLC	=	High-Performance Liquid Chromatography
LC/MS	=	Liquid chromatography-mass spectrometry
LOD	=	Limit of Detection
LOQ	=	Limit of Quantitation
GI	=	Gastro-Intestinal
MTD	=	Maximum Tolerated Dose
AUC	=	Area under the plasma concentration versus time curve
$t_{1/2}$	=	Half-life
C_{max}	=	Peak Concentration
Cl_{oral}:	=	Apparent oral clearance
S.E.M	=	Standard Error of the Mean

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3.7 Tables

Table 3.1. (A) Average extraction efficiencies of aPPD in plasma and tissues. (B) Pharmacokinetic parameters for aPPD following oral administration of 70 mg/kg (n = 3). Values are reported as mean ± SEM.

A

Plasma (%)	Stomach (%)	Small intestine (%)	Liver (%)	Lung (%)	Spleen (%)	Kidney (%)	Brain (%)
59.0±2.4	77.5±4.7	94.9±9.8	91.7±6.9	89.7±12.6	95.4±12.4	104.6±6.1	110.0 ± 2.7

B

Body Weight (g)	$t_{1/2}$ (min)	C_{max} (μg/ml)	Cl_{oral} (ml/min/kg)	AUC (μg.min/ml)
26.4 ± 2.9	28.5 ± 16.7	3.9 ± 1.4	195.4 ± 32.4	330.7 ± 51.9

$t_{1/2}$: Half-life
C_{max}: Peak Concentration
Cl_{oral}: Apparent oral clearance
AUC: Area under the plasma concentration versus time curve

3.8 Figure legends

Figure 3.1. (A) Chemical structure of aPPD. (B) Stability of aPPD in 100% ethanol for 28 days. Conditions are 4°C (empty circles), room temperature (R.T., filled squares), and 60°C (filled triangles), n = 3. (C) Mean plasma concentration-time profile of aPPD in mice following oral administration of 70 mg/kg, n = 3 per time point. Values are reported as mean ± SEM.

Figure 3.2. % administered dose of aPPD in specified tissues isolated from mice following oral administration of 70 mg/kg. (A)-(D): (A) 30 minutes (B) 60 minutes (C) 120 minutes (D) 200 minutes. (E) % administered dose of aPPD in the brain at 30, 60, 120, and 200 minutes time points. Results are expressed as mean values ± SEM. n = 3.

Figure 3.3. (A) *In vivo* efficacy of aPPD in PC-3 bearing nude mice. Changes in tumor volume was followed over time for animals treated with the oral gavage vehicle control (filled circle, ethanol, propylene glycol and water), saline (empty circle), aPPD (70 mg/kg QD x 5; filled triangle), and docetaxel (20 mg/kg Q7D x 4; filled square). Average tumor volumes are expressed as a percentage of the average initial tumor volume of each group at day 14, post PC-3 cells inoculation. Mean value ± SEM is shown with error bars. A statistically significant difference was found between aPPD and the control (p=0.0044), saline (p=0.0159), and docetaxel (p=0.0148) on day 39. n = 8 for aPPD and docetaxel. n = 6 for the oral gavage control and saline. (B) *In vivo* toxicity as assessed by decrease in mean body weight. Body weight loss of > 20% is considered severe enough to warrant termination of the animals. No animals showed any signs of toxicity other than weight loss in these studies. Docetaxel showed a significant weight loss compared to saline (p=0.0001). n = 8 for aPPD and docetaxel. n = 6 for the oral gavage control and saline. Mean value ± SEM is shown with error bars. (C) Representative tissue microarray spots for PC-3 tumors: immunostained using a TUNEL assay. Apoptotic cells

could be identified in areas of viable tissue, as indicated by the arrows. (D) Apoptotic index summarized from ApopTag® staining of tissue microarrays. (E) summarized Ki-67 staining of tissue microarrays. Mean scores were determined as a percentage of the total number of cells. Four cores of tissue were extracted per tumor and one tumor per animal. n (total number of cores) = 24 for the control and saline and 32 for aPPD and docetaxel. Mean value ± SEM is shown with error bars. * signifies statistically significant difference from the control.

3.9 Figures

Figure 3.1

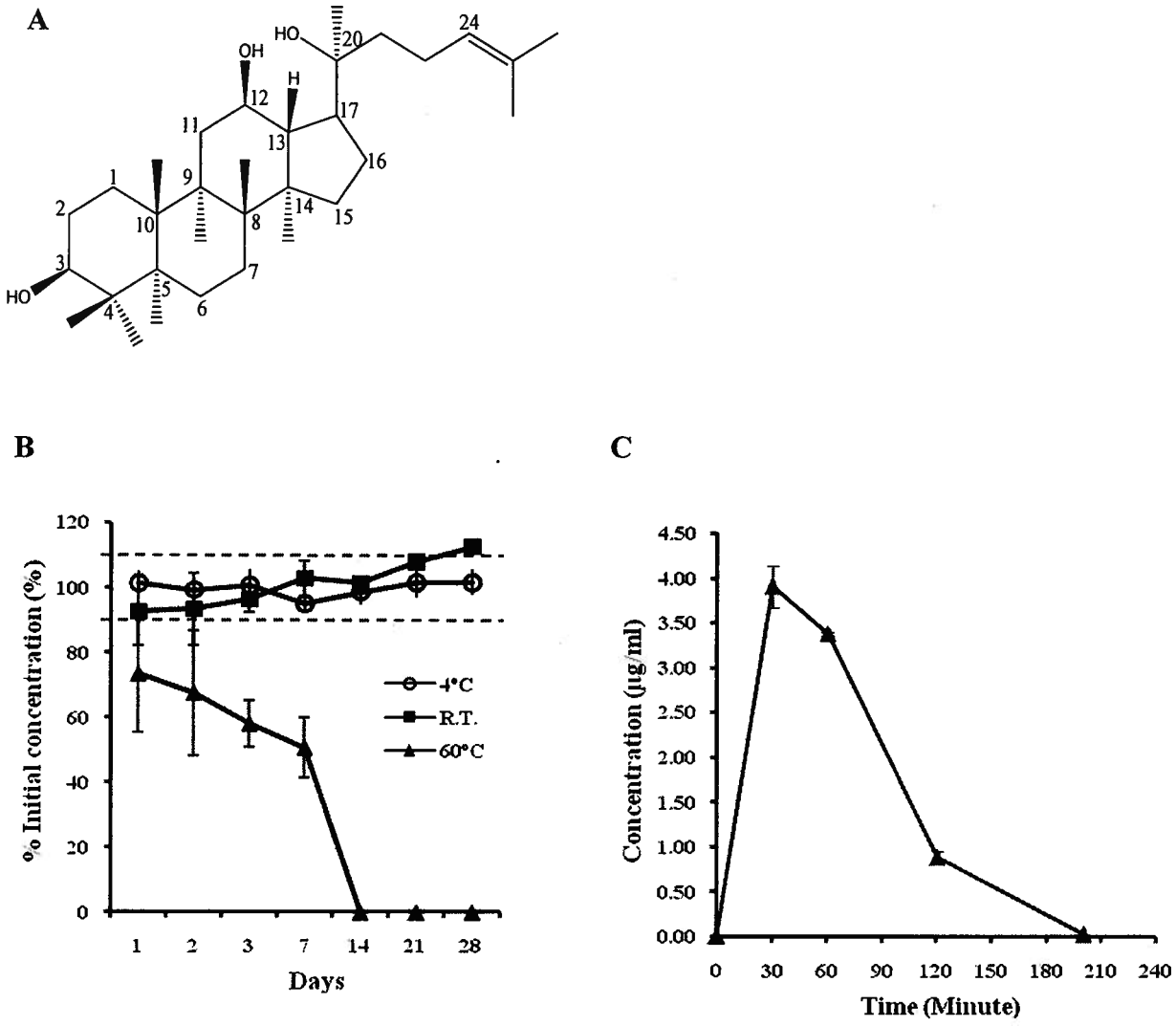
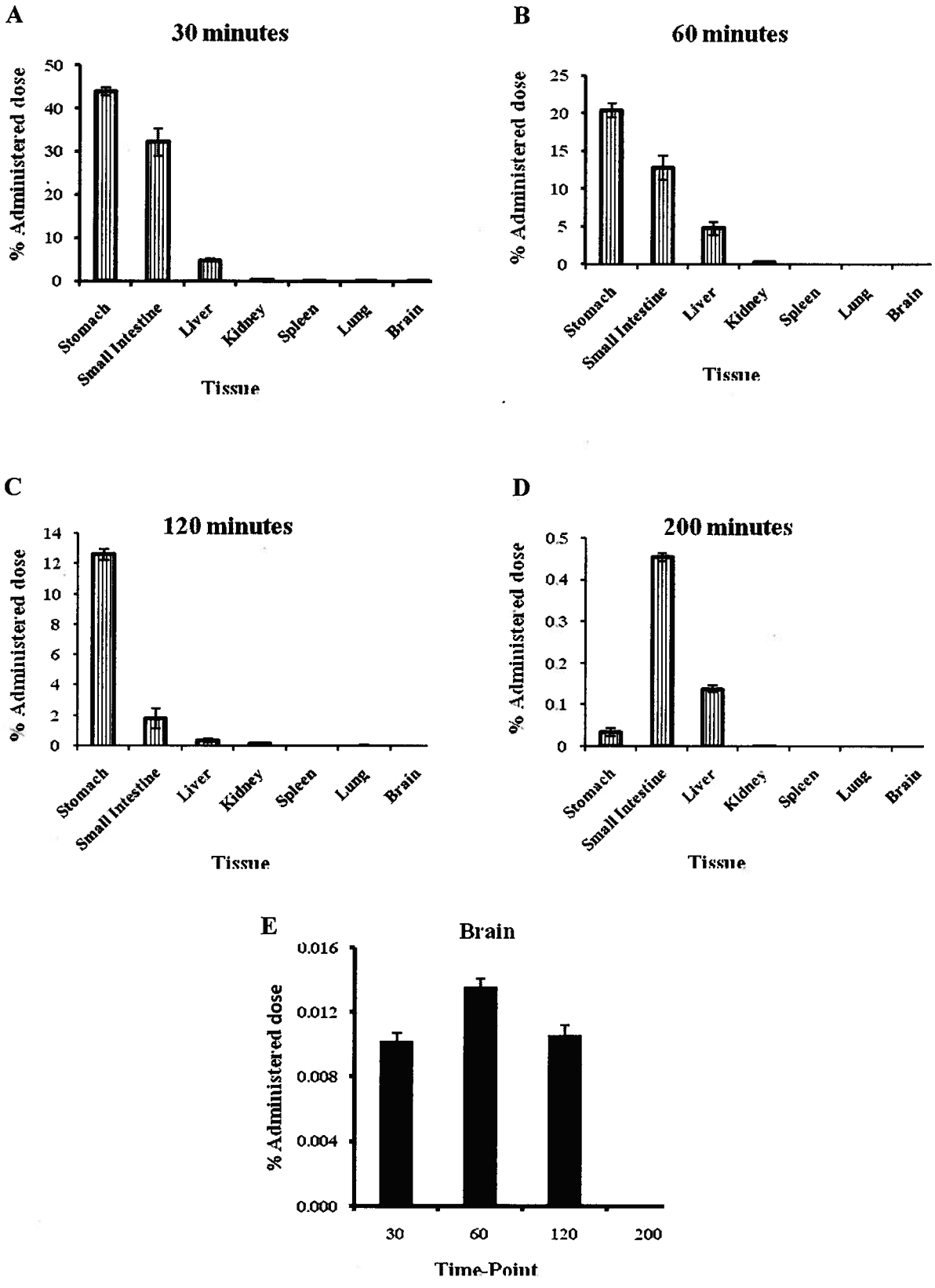


Figure 3.2


A
30 minutes
% Administered dose
50
40
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Stomach
Small Intestine
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60 minutes
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25
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Liver
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0.3
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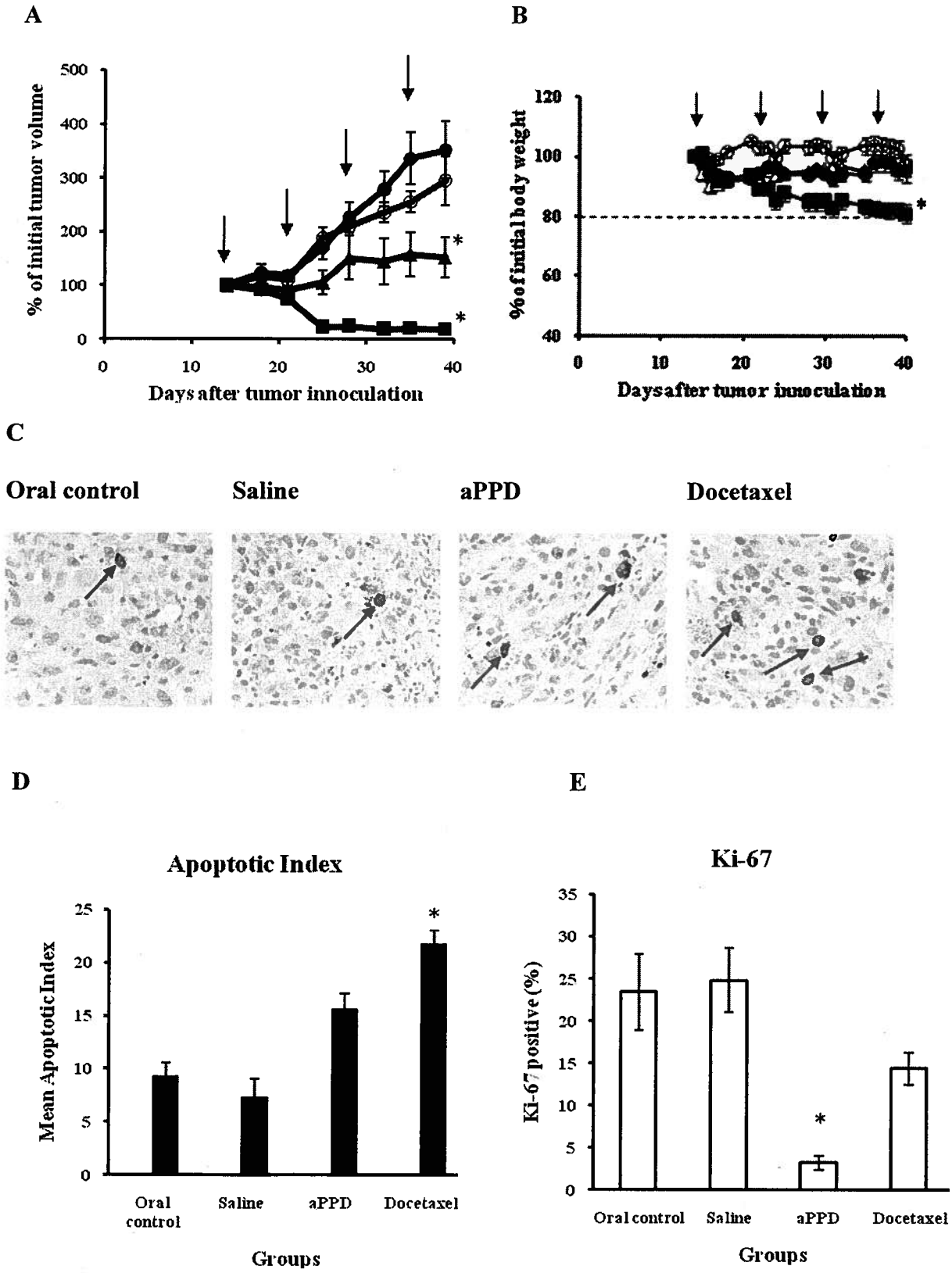
Figure 3.3


A
% of initial tumor volume
Days after tumor innoculation
B
% of initial body weight
Days after tumor innoculation
C
Oral control
Saline
aPPD
Docetaxel
D
Apoptotic Index
Mean Apoptotic Index
Oral control
Saline
aPPD
Docetaxel
Groups
E
Ki-67
Ki-67 positive (%)
Oral control
Saline
aPPD
Docetaxel
Groups

4. THIRD MANUSCRIPT CHAPTER

Rh2 or its aglycone aPPD in Combination Therapy with docetaxel[1]

[1] A version of this chapter has been submitted for publication. Co-authors: Andy Eberding, Ladan Fazli, Antonio Hurtado-Coll, William Jia, Euan Ramsay, Marcel B. Bally, and Emma Tomlinson Guns.

4.1 Introduction

The decline in prostate cancer mortality is partly attributed to the increased use of androgen deprivation treatment during the initial stage of the disease and by use of maximum androgen blockade strategies at advanced stage (Collin *et al*, 2008). These medical interventions often eradicate androgen-dependent tumor cells but have little or no impact on androgen-independent cells which can then repopulate the tumor (Pienta, 2001). Surprisingly there are few chemotherapeutic drugs that are considered highly effective when used to treat prostate cancer patients that have relapsed and/or have metastatic disease. In recent years, however, new drugs and drug combinations and particularly those involving taxanes have contributed to improvements in treatment outcomes in prostate cancer (de Wit, 2008; Oh, 2003). It has been suggested that optimal drug combination effects will be achieved when combining drugs which have different mechanisms of activity and different toxicity profiles (Chu & Devita, 2005). In addition, drug combinations that interact in a manner that result in synergy or drug potentiation (i.e. where the combined effects are far greater than that expected on the basis of the effects of the individual agents) are desirable (Ramsay *et al*, 2005). Such drug combinations are of particular interest since many targeted highly specific agents may not exhibit sufficient activity when administered as single agents, but when used in combination with current standards of care may provide meaningful improvements in therapeutic outcomes. This rationale will also be applicable in the context of complementary and alternative medicines (CAMs); agents that are being used by patients with the belief that they may augment treatment responses achieved by conventional therapy.

Patients that use CAMs do so on the assumption that the agents are well tolerated and safe (Richardson *et al*, 2000b), however this assumption needs to be questioned and evaluated

scientifically. Our research team has been testing the hypothesis that combinations of well tolerated and therapeutically active phytochemicals (specifically Rh2 and aPPD) derived from *Panax Ginseng* C.A. Meyer can be combined with docetaxel to achieve enhanced drug efficacy in models of prostate cancer. Enhanced therapeutic activity should occur without additional toxicity, particularly if the agents used interact in a manner that results in drug-drug synergy. Inherent to the concept of synergy, comparable therapeutic effects of single agents should be achieved at substantially lower doses when the selected drugs are used in combination. This is reflected in a measured parameter called the dose reduction index (DRI). In order to measure drug combination effects and to determine DRIs, it is first necessary to establish the activity of the single agents and then compare these activities to those achieved when the drugs are used in combinations. Although there are many ways to assess combination effects, a commonly used method relies on the median effect principle first described by Chou and Talalay (Chou & Talalay, 1984). Methodology developed around this analysis method uses a fixed drug-drug ratio experimental design and the resultant data can be analyzed with the help of a commercially available software package, Calcusyn.

We have previously developed oral formulations of ginsenoside Rh2 and its aglycone aPPD. These ginseng metabolites were bioavailable following oral administration and, following daily administration at the maximum achievable dose, could delay progression of established PC-3 tumors in mice (manuscripts submitted). Rh2 and aPPD were well tolerated and in this castration-resistant prostate cancer model the activity of Rh2 was comparable to that achieved with single agent docetaxel (used as a positive control). Here we report on studies assessing *in vitro* and *in vivo* combinations of Rh2 or aPPD with docetaxel. Therapeutic activity of the agents alone and in combination were determined *in vitro*; where therapeutic effects were

measured using a single metabolic assay endpoint (MTS assay) in four prostate cancer cell lines: PC-3 (androgen-independent, bone metastasis), LNCaP (androgen-dependent, lymph node metastasis), DU145 (androgen-independent, brain metastasis), and C4-2 (androgen-independent, derived from LNCaP) (Krueckl *et al*, 2004; Van Bokhoven *et al*, 2003). *In vivo* combinations of Rh2 or aPPD with docetaxel were assessed in mice bearing established PC-3 tumors. Combinations of Rh2 or aPPD with docetaxel were effective and well tolerated in mice and the *in vitro*/*in vivo* results indicated that combinations produced interactions that were additive or synergistic.

MATERIALS AND METHODS

Test compounds and materials

Ginsenoside Rh2 (M.W. 640.89 g/mol) and its aglycone aPPD (M.W. 460 g/mol), as a white powder, were purchased from LKT Laboratories Inc., St. Paul, MN. (USA), or generously supplied by Panagin Pharmaceuticals Inc, Richmond, BC (Canada). Taxotere® (docetaxel) was purchased from B.C. Cancer Agency Pharmacy as a 40 mg/ml solution in polysorbate 80, manufactured by Aventis Pharma Inc. Saint-Laurent, Québec (Canada). Cholic acid (M.W. 408.58 g/mol) and all other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

In vitro activity as determined using the MTS metabolic activity assay

All cell lines (PC-3, LNCaP, DU145, and C4-2) were obtained from ATCC, Rockville, MD. PC-3 and DU145 were cultured in DMEM medium supplemented with 5% fetal bovine serum (FBS). LNCaP and C4-2 were cultured in RPMI medium supplemented with 10% FBS. Cells were grown in 10 cm dishes and maintained at 37°C, 5% CO_2 and 90% relative humidity. Cells were seeded in triplicate at a density of 5,000 cells/well onto 96-well plates and after 24 hours, the media was aspirated and replaced with Rh2, aPPD, docetaxel, Rh2+docetaxel, or aPPD+docetaxel prepared at the appropriate concentrations in serum containing media (100μL). After a 72 hour incubation, MTS solution (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega, WI, USA) was added directly to wells, incubated for 1–4 hours and the optical density read at 490 nm absorbance with a 96-well plate reader (Bio-Tek Instruments Power Wave X, Winooski, VT, USA). The IC_{50} (the concentration required to achieve a 50% reduction in MTS labeling compared to controls) of the agents against these cell lines was then estimated using the Calcusyn software package (Biosoft, Cambridge, UK). These experiments were

repeated at least three times. When using drug combinations, docetaxel was combined with Rh2 or aPPD according to the constant ratio combination experimental design. To define this fixed ratio docetaxel was combined with Rh2 or aPPD in a ratio equivalent to IC_{50}/IC_{50} ratios (Table 4.1). For Rh2:docetaxel or aPPD:docetaxel ratios of 1000:1 (LNCaP), 100:1 (PC-3, C4-2), and 25:1 (DU145) were used. Docetaxel was mixed with Rh2 or aPPD at the indicated ratios and at a concentration that was 8-fold greater then the individual IC_{50}s. This solution was then serially diluted to achieve 8 effective doses.

Combination Index (CI) and Dose Reduction Index (DRI)

Data from the metabolic assay was then analyzed using the Calcusyn software package to assess drug-drug interactions based on the Median Effect Principle (MEP) developed by Chou and Talalay. This method is derived from the law of mass action and enzyme kinetic models (Chou & Talalay, 1984). The MEP equation is represented by $\frac{f_a}{f_u} = \frac{D}{(D_m)^m}$, where f_a is the fraction affected by the drug, f_u is the unaffected fraction (1-f_a), D is the dose of the drug, D_m is the median-effect dose (signifies drug potency) and m is an exponent signifying the shape of the dose-effect curve. This equation determines whether the effect of a drug combination is greater than expected (synergistic) based on the effect of the single agents. The MEP can also be used to define the combination index (CI) function, calculated using $CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$ (for mutually exclusive drugs that have the same or similar modes of action) where D_1 and D_2 are the doses of drug 1 and drug 2. D_x refers to the dose of drug required to produce an effect of x percent. Thus a CI can be calculated for a given combination and effect level; where a CI < 1 indicates synergy, a CI > 1 indicates antagonistic interactions and a CI = 1 indicates additive effects. The MEP is also used to obtain the Dose Reduction Index (DRI). DRI is a measure of the extent to

which the dose of each drug in a given combination is changed to achieve a given effect level (e.g. IC_{50}) relative to the dose of the individual drugs required to achieve the same effect level.

$DRI = \frac{D_x}{D_c}$, where D_x is the single agent drug dose required to produce an effect of x percent (e.g. 50% reduction in MTS labeling). D_c is the dose of the drug in a combination that generates the same effect.

Oral gavage formulation

Rh2 and aPPD were formulated for oral administration as described previously (Manuscripts submitted). In brief, Rh2 (232 mg) or aPPD (120 mg) were dissolved in 1.8 ml of 100% ethanol and mixed vigorously (based on pre-determined maximum solubility of 130 and 68.4 mg/ml for Rh2 and aPPD, respectively). In a separate container 700 µl of ddH_2O was mixed (by vortexting) with 5.5 ml of propylene glycol. The propylene glycol-water mixture was then mixed with the Rh2 or aPPD containing ethanol solution. The ethanol solubilized Rh2 and aPPD were stable for at least 1 month at 4°C and the formulations for oral administration were prepared just prior to administration.

***In vivo* studies**

PC-3 cells (1.0×10^6) were inoculated subcutaneously into 6-8 week old male nude mice (Harlan Sprague Dawley, Inc.) weighing 25-31 g. After 14 days, the developing tumors were measured and mice randomly assigned to different treatment groups. A caliper was used to measure the three perpendicular axes of each tumor. The formula $V = (L \times W \times H)\frac{\pi}{6}$ where L is the length, W the width, and H the height, was used to calculate the tumor volume (Brigham *et al*, 1978). A minimum tumor volume of 100 mm^3 was required for a given mouse to be included in the study. Mice were monitored daily, weighed once a week, and tumor size was determined two times per

week. The mice were monitored daily for changes in weight and other signs of acute toxicity such as lethargy and disorientation.

To define a dose response curve for docetaxel in the PC-3 model, seven different treatment groups were defined and drug was administered intravenously (via the lateral tail vein) using a Q7D x 4 dose schedule: (i) Docetaxel – 10 mg/kg (83-103 µl (3.0 mg/ml solution)); (ii) Docetaxel – 12 mg/kg (100-124 µl (3.0 mg/ml solution)); (iii) Docetaxel – 14 mg/kg (117-141 µl (3.0 mg/ml solution)); (iv) Docetaxel – 16 mg/kg (80-99 µl (5.0 mg/ml solution)); (v) Docetaxel – 18 mg/kg (90-112 µl (5.0 mg/ml solution)); and (vi) Docetaxel – 20 mg/kg (100-124 µl (5.0 mg/ml solution)). The seventh group was treated (iv) with saline as a control. These data were used to define a docetaxel dose that produced a therapeutic effect that was measurable, but significantly below the maximum tolerated (or maximum therapeutic) dose of docetaxel. This dose was used in the combination studies with Rh2 and aPPD.

For *in vivo* efficacy studies assessing combination effects seven treatment groups were defined: (i) Rh2 solubilized in ethanol:propylene glycol:water (2:7:1 v/v ratio) was administered by oral gavage at a dose of 120 mg/kg (103-128 µl) using a QD x 5 schedule each week for 4 weeks; (ii) aPPD solubilized in ethanol:propylene glycol:water (2:7:1 v/v ratio) was administered by oral gavage at a dose of 70 mg/kg (117-145 µl) using a QD x 5 schedule each week for 4 weeks; (iii) oral control consisting of ethanol:propylene glycol:water (2:7:1 v/v ratio) given at a dose of 120 mg/kg (103-128 µl) using a QD x 5 schedule; (iv) Docetaxel dosed alone, as a treatment control, was administered intravenously via the lateral tail vein at a dose of 10 mg/kg (83-103 µl) using a Q7D x 4 dose schedule; (v) Rh2+docetaxel (Rh2 – 120 mg/kg QD x 5 per week, docetaxel – 10 mg/kg Q7D x 4); (vi) aPPD+docetaxel (aPPD – 70 mg/kg QD x 5 per week; docetaxel – 10 mg/kg Q7D x 4); and (vii) saline (83-103 µl) given Q7D x 4

Analysis of apoptosis and proliferation markers

At the end of the efficacy studies (39 days after tumor cell inoculation) mice were sacrificed and tumors were excised, formalin-fixed, and then paraffin-embedded. A Tissue Micro-Array (TMA) was constructed by extracting four 600-μm diameter cores of tumor tissue from each paraffin block using a Beecher Instruments tissue core extractor and re-embedding these cores into a gridded paraffin block. After construction, 4-μm tissue sections were cut and adhered to Fisher SuperFrost Plus glass slides. Apoptotic cells were then visualized by terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) staining which was carried out using the ApopTag® Peroxidase Kit. Tissues were pretreated with proteinase K for 15 minutes. Subsequently, TdT enzyme was applied and the tissue incubated in a humid chamber at 37°C for 1 hour. Anti-digoxigenin peroxidase conjugate was later applied for 30 minutes, followed by DAB, and counter-stain with hematoxylin, as well as Staining Blueing Reagent. For Ki-67, a Mouse-To-Mouse immunohistochemical detection kit (Chemicon®) was used to decrease nonspecific staining. Ki-67 (Dako, Carpinteria, California) (1:200) monoclonal antibodies were used in combination with immunoperoxidase procedures (LSAB + peroxidase kit). Antigen retrial was applied by steaming with citrate buffer for 30 minutes. The TMA slides were imaged digitally and evaluated by visual scoring of apoptotic and Ki-67 positive cells. Scores from individual tumor cores with significant necrosis were omitted. Scoring was completed by two pathologists blinded to the study groups.

Statistical analysis

For each studied variable, mean and standard error of the mean (S.E.M.) were calculated. Statistical significance and differences between the described treatment groups were assessed

using the Tukey-Kramer/Student's t-tests from within One-way Analysis of Variance (ANOVA) analysis. For these tests, the level of significance was set at a p value of < 0.05. All mean values were reported as mean ± S.E.M.

RESULTS:

Cell viability and IC_{50}

In order to assess drug-drug interactions *in vitro*, the IC_{50} of the single agents were determined first. The IC_{50} of Rh2, aPPD and docetaxel against the four prostate cancer cell lines (PC-3, LNCaP, DU145, and C4-2) are summarized in Table 4.1. The IC_{50} of Rh2 varies from 6.6 ± 0.27 μM against DU145 cells to 12.8 ± 1.22 μM against LNCaP cells. The IC_{50} range for aPPD is between 5.6 ± 0.40 μM against DU145 cells and 12.6 ± 1.66 μM against LNCaP cells. Docetaxel IC_{50} varying between 0.017 ± 0.002 μM against LNCaP cells and 0.25 ± 0.08 μM against DU145 cells. The androgen-independent cell lines were consistently more sensitive to the phytochemicals Rh2 and aPPD when compared to the androgen-dependent LNCaP cell line. The LNCaP cells, however, were most sensitive to docetaxel.

Assessment of drug-drug interations

Assessments of *in vitro* drug-drug interactions were completed using the fixed ratio experimental design methodology described in the Methods. The calculated combination indices determined at the IC_{50}, IC_{75} and IC_{90} have been summarized in Figure 4.1. When using the androgen independent cell lines (PC-3, DU145, and C4-2 cells (Figure 4.1A, C, and D), combinations of Rh2 or aPPD with docetaxel exhibited CI values mostly equal or below 1 at the doses needed to produce 50%, 75% and 90% decreases (relative to controls) in MTS labeling. This suggested that the drug interactions resulted in synergy based on the metabolic endpoint measured by the MTS assay. In contrast, when using the androgen-dependent LNCaP cells combinations of Rh2 or aPPD with docetaxel resulted in CI values of greater then 1 at doses required to achieve 75% and 90% decreases (relative to controls) in MTS labeling (Figure 4.1B). These data suggest that

drug-drug interactions are slightly antagonistic when using Rh2 or aPPD with docetaxel *in vitro* against LNCaP cells.

The drug-drug interaction assessments completed with the Calcusyn program also estimate Dose Reduction Indices and these have been summarized in Figures 4.2 and 4.3. When using drug combinations that result in synergistic interactions, significantly lower drug doses (compared to those used when the drugs are added alone) are required to achieve a specific effect level. As indicated by the data summarized in Figures 4.2 A, C and D for the androgen-independent prostate cancer cell lines, in the presence of docetaxel, the amount of the phytochemical Rh2 or aPPD required to achieve a 50 decrease (relative to controls) in MTS labeling is reduced between 4- to 21-fold. For example, for the androgen-independent cell line C4-2 (a cell line derived from LNCaP cells) the IC_{50} of aPPD alone is 5.6 μM, while in the presence of docetaxel the dose required to achieve the same effect level is 10-fold lower (0.5 μM). In contrast when the interactions are antagonistic, as observed for the LNCaP cells treated with combinations of aPPD and docetaxel (Figures 4.2B), aPPD must be added at concentrations that are 1.5 fold greater than that needed to achieve the same effect when aPPD is used alone. A similar analysis can be done for docetaxel, where one can determine whether less or more docetaxel is required to achieve a specific effect level when the drug is used in combination with Rh2 or aPPD, and these DRI data are summarized in Figure 4.3. Again for the androgen-independent prostate cancer cell lines, substantially lower docetaxel doses are required to achieve a 50 decrease (relative to controls) in MTS labeling (Figures 4.3A, C and D). Focusing again on the C4-2 and LNCaP cell lines, in the presence of aPPD the IC_{50} of docetaxel for C4-2 cells is 0.005 μM, which is 10-fold lower than the IC_{50} of docetaxel when used as a single agent

(0.048 μM) and, in contrast, in the presence of aPPD the IC_{50} of docetaxel for LNCaP cells is actually slightly higher then in the absence of aPPD (Figure 4.3B).

***In vivo* anti-PC-3 tumor activity of docetaxel alone and in combination with Rh2 and aPPD**

The results thus far indicate that Rh2 or aPPD can be combined with docetaxel to produce synergistic interactions *in vitro*, as judged by a single endpoint assay measuring metabolic activity, when used against androgen-independent cell lines. Further, previous studies from our group have shown that Rh2 and aPPD are active as orally administered (QD x 5 each week for 4 weeks) single agents *in vivo* against tumors which arise following s.c. injection of the androgen-independent PC-3 cells (manuscripts submitted). To determine whether the interactions between docetaxel and Rh2 or aPPD resulted in improved therapeutic effects *in vivo*, the activity of the drugs alone and in combination were determined in nude mice bearing established (100-150 mm^3) s.c. PC-3 tumors. It is known that docetaxel is effective when used to treat mice bearing PC-3 tumors, particularly when used at its maximum tolerated dose. When assessing drug combination effects *in vivo* it is important to select doses where maximal activity is not observed in order to determine whether the second agent used in a combination actually contributes additional therapeutic effects. For this reason an extensive dose escalation study was completed with docetaxel (10-20 mg/kg) in mice bearing established PC-3 tumors. These results are summarized in Figures 4.4A and B. The results indicate that docetacel is active at all doses used. On day 39, the average tumor volume for saline treated animals was approximately 7 times the size of the average tumor mass determined when treatment was initiated. Tumors from animals treated with docetaxel were 132, 113, 114, 84, 62, and 37% of the initial volume when treated at doses of 10, 12, 14, 16, 18, and 20 mg/kg, respectively. On day 39, the mean tumor size from

animals treated at a dose of 10 mg/kg was statistically different from the 20 mg/kg (p=0.0006) and saline (p=0.0001) groups. It should be noted that in animals treated at a dose of 20 mg/kg there was significant (>20%) body weight loss; indicating that this is the maximum tolerated dose in these tumor bearing mice. Less than 7% body weight loss was noted in animals treated at the 10 mg/kg dose (Figure 4.4B). Based on these data the 10 mg/kg dose was used to assess combination effects with Rh2 and aPPD.

The therapeutic activities of Rh2 (120 mg/kg QD x 5 every week for 4 weeks), aPPD (70 mg/kg QD x 5 every week for 4 weeks), docetaxel (10 mg/kg Q7D x 4), Rh2+docetaxel, and aPPD+docetaxel were determined in a study that included saline and the ethanol:propylene glycol:water (2:7:1 v/v) vehicle control. These results, summarized in Figures 4.4C-E, demonstrate that docetaxel, Rh2+docetaxel, and aPPD+docetaxel were highly effective in inhibiting PC-3 tumor growth. On day 39, the average tumor volume for saline treated and oral control treated animals was approximately 7 to 8 times the size of the average tumor volume determined when treatment was initiated. For animals treated with Rh2 and aPPD, the tumor volume were 2 to 3-fold greater then the average tumor volume determined when treatment was initiated and the tumors at this time were statistically smaller then those tumors from animals treated with the oral vehicle control (p=0.0071 for Rh2 and p=0.0215 for aPPD). Docetaxel administered at the 10 mg/kg dose inhibited tumor growth significantly and the tumor size on day 39 was just slightly larger then the average tumor volume determined when treatment was initiated. It is notable that when docetaxel was combined with Rh2 or aPPD, the tumors actually regressed. For combinations comprising Rh2+docetaxel the tumors were 15% smaller than the average tumor volume determined when treatment was initiated and for combinations of aPPD+docetaxel the tumors were 27% smaller than the average tumor volume determined when

treatment was initiated (Figure 4.4D). At the end of the study (day 39), there was no statistically significant difference in tumor volume of animals treated with docetaxel alone compared with animals treated with Rh2+docetaxel (p=0.0614). Tumors from animals treated with aPPD+docetaxel were statistically smaller then those tumors from animals treated with docetaxel alone (p=0.0482). The combined effects of docetaxel administered at 10 mg/kg and Rh2 or aPPD were equivalent to those effects measured when using 16 to 18 mg/kg docetaxel (refer to Figure 4.4A). Improved therapeutic effects at the 10 mg/kg dose of docetaxel were associated with small, albeit not significant, increases in weight loss (Figure 4.4E).

PC-3 tumors were isolated from mice at the end of the study described above and were prepared for immunohistochemical assessment of apoptosis and Ki-67. These results are summarized in Figure 4.5. Tumors from mice treated with docetaxel as a single agent exhibited the highest apoptotic index (41.3%) (Figures 4.5 A and B). Apoptotic indices were 32.5 and 29.7% for tumors from mice treated with combinations of aPPD+docetaxel and Rh2+docetaxel, respectively. The average apoptotic index for the control tumors were 9.1 and 6.85% for the oral control and saline respectively. Tumors from animals treated with Rh2 or aPPD alone exhibited apoptotic indices of 23.3% and 27.2%, respectively. Apoptotic indices determined for tumors isolated from mice in all treatment groups were statistically significantly different from the control groups. Animals treated with docetaxel alone exhibited a mean apoptotic index that was significantly greater then that determined in tumors from animals treated with Rh2+docetaxel (p=0.0074) but not from tumors from animals treated with aPPD+docetaxel (p=0.1156). There was not a significant difference when comparing the apoptotic indices in tumors from animals treated with Rh2, aPPD, Rh2+docetaxel, and aPPD+docetaxel. Ki-67 results, summarized in Figure 4.5C indicate that the greatest labeling occurred in tumors from animals treated with the

oral control (30.3%), saline (29.0%) and in tumors from animals treated with docetaxel (15.2%). The lowest labeling was oobserved in tumors from mice treated with aPPD+docetaxel (6.0%) and Rh2+docetaxel (7.0%). Ki-67 staining levels in tumors from mice treated with aPPD+docetaxel, Rh2+docetaxel, Rh2, and aPPD were all significantly different from the control with p values ≥ 0.0001. When compared to results obtained in tumors from animals treated with docetaxel, the Ki-67 labeling in tumors from mice treated with Rh2 (p=0.0092), aPPD (p=0.0407), Rh2+docetaxel (p=0.0003), and aPPD+docetaxel (p=0.0005) were all statistically lower.

DISCUSSION:

Previous studies assessing the therapeutic effects of novel oral formulations of ginsenoside Rh2 and its aglycone aPPD indicated that at the highest achievable dose (limited due to gavage volume limitations imposed by the institutional animal care committee), the systemic blood levels achieved following daily gavage were sufficient to delay growth of established PC-3 tumors. These studies indicated that the orally dosed ginsenosides were bioavailable and effective. The treatment effect of Rh2 was not significantly different then those obtained when using docetaxel administered at its MTD, albeit it was clear that docetaxel was therapeutically superior. It was also clear that docetaxel was significantly more toxic then either Rh2 or aPPD. Since it has been argued that CAMs can be used to augment the therapeutic activity of conventional drugs without risks of additional or new toxicities (Richardson *et al*, 2000a), it was reasonable to ask whether isolated ginseng metabolites would enhance or inhibit the therapeutic effects of docetaxel. In terms of CAMs and their use in prostate cancer patients, focusing on components isolated from *Panax Ginseng* C.A. Meyer was also appropriate considering extracts from this ginseng root have been promoted as being antioxidative and anticarcinogenic (Nah *et al*, 1995; Yun *et al*, 2001; Zhang *et al*, 1996). Hence the studies developed here were designed to determine whether Rh2 or aPPD when combined with docetaxel would improve treatment outcomes in a manner that was not associated with overt increases in toxicity. Such an outcome is most likely achieved when using drugs in a combination that interact synergistically. The results summarized here clearly suggest that in prostate cancer cell lines that have been defined as androgen-independent, docetaxel and Rh2 or aPPD interact in a manner that results in at least additive and more likely synergistic interactions.

In vitro, docetaxel IC_{50} values against PC-3, LNCaP, DU145, and C4-2 cells were comparable to other investigators (Budman *et al*, 2002; Fulzele *et al*, 2007; Shigemura *et al*, 2007; Van Bokhoven *et al*, 2003) (Table 4.1). In PC-3 and LNCaP cell lines, Wang et al. (Wang *et al*, 2007) obtained IC_{50} values of more than twice our results for Rh2 and aPPD, following a 72 hour incubation. Although underlying causes of differences in IC_{50} values are complex, it has been shown that significant differences in passage numbers may lead to significant behavioral differences in many cell types, including LNCaP cells (Esquenet *et al*, 1997; Hughes *et al*, 2007).

As mentioned earlier, both Rh2 and aPPD have been reported to inducing G1 cell-cycle arrest (Oh *et al*, 1999; Popovich & Kitts, 2002). Docetaxel has been shown to increase the activity of the cdk1/cyclin B1heterodimer by blocking cyclin B1 degradation and prolonging cdk1 activation (Gomez *et al*, 2006). Treatment of cells with Rh2+docetaxel or aPPD+docetaxel should present the majority of cells with the predominant possibility of G1 cell-cycle arrest, considering the significantly higher ratios of the ginsenosides in these combinations. Cells exposed to docetaxel would experience inhibition of microtubule dynamic instability via binding to B-tubulin, cell cycle G2/M phase transition and mitotic arrest, culminating in apoptosis (Bhalla, 2003). Cells undergoing G1 cell-cycle arrest might not progress to G2/M phase where docetaxel induced mitotic arrest would occur and vice versa. This scenario is most conducive to synergistic and additive activities as observed with Rh2+docetaxel and aPPD+docetaxel combinations (Figure 4.1A, C, and D (IC_{50} and IC_{75}). Combinations of Rh2+docetaxel and aPPD+docetaxel when used to treat LNCaP cells appeared to produced antagonistic effects and its difficult to explain this, especially since the combinations appeared to be synergistic when used to treat C4-2 cells (an androgen-independent cell line derived from LNCaP cells). It is

particularly interesting that antagonism was only observed in cell lines expressing the functional p53. LNCaP cells express the functional tumor suppressor protein p53 while PC-3 is p53 deficient (Wang *et al*, 2008). C4-2 expresses the functional p53 protein albeit at a lower level then that seen in the parent LNCaP cells (Ko *et al*, 1996). The wild-type alleles of p53 are commonly lost or mutated during advanced tumor development (Scott *et al*, 2003). One could therefore speculate that in LNCaP cells, stress induced by ginsenoside treatment such as increase in DNA fragmentation associated with aPPD treatment (Popovich & Kitts, 2002), would trigger the p53-p21-cdk2 inhibition pathway, causing G1 cell cycle arrest, a mechanism that would be comparable to that observed when using ginsenosides alone. As would be expected, drugs exhibiting comparable mechanisms of activity induce combination effects that are no better than additive and more often than not antagonistic. Importantly, in clinical investigations with breast cancer patients, inhibition of cdk1 has been associated with a decrease in taxane-induced apoptosis, suggesting a pro-apoptotic mediation role for cdk1 (Castedo *et al*, 2002). Simultaneous activation and inhibition of cdk1 in prostate cancer cells *in vitro* seems to cause antagonism, as demonstrated by Gomez et al. following treatment of DU145 cells with docetaxel and flavopiridol, a known cdk1 inhibitor (Gomez *et al*, 2006). Since both Rh2 and aPPD are known to suppress cdk2 activities (Ota *et al*, 1997; Wang *et al*, 2008), it would be of great interest to investigate whether certain levels of cdk2 downregulation could lead to antagonistic outcomes, when combined with docetaxel. It is also known that docetaxel acts differently depending on whether the prostate cancer cell line is androgen-dependent or independent, and whether the cell lines express the androgen receptor (Gomez *et al*, 2006). The results here indicated that LNCaP cells are 3- to 15-fold more sensitive to docetaxel (Table 4.1). Only C4-2 and LNCaP express the androgen receptor (Krueckl *et al*, 2004) and these are the cell lines that

are most sensitive to docetaxel while PC-3 and DU145 express little or no androgen receptor (Van Bokhoven *et al*, 2003). It would be useful to investigate the effect of androgen receptor expression and the role of p53 expression following treatment with docetaxel alone and in combination with selected ginsenosides.

Previous studies from this lab have shown that Rh2 and paclitaxel could be combined to produce synergistic effects in LNCaP cells but the fixed ratio used in these studies was 1:1 rather then 1000:1 used here with docetaxel (Xie *et al*, 2006). Although docetaxel exhibits 1.3 to 12-fold greater cytotoxicity in several cell lines compared with paclitaxel the differences would not be accounted for the 1000 fold difference in drug:drug ratios used in these different studies. Recent studies have suggested that drug:drug interactions can be highly sensitive to the drug:drug ratio used (Mayer & Janoff, 2007) and this variable as well as many other variables known to influence combination effects were not explored here.

Drug combinations involving cytotoxic drugs in clinical settings are motivated primarily by the prospect of achieving significant efficacy with reduced associated toxicity. Drug combinations yield cost benefits also as the doses of drugs required to elicit a specific therapeutic benefit as part of a combination are typically lower than when dosed alone. Although it is difficult to predict *in vivo* combination outcomes on the basis of *in vitro* results, the *in vitro* DRI offers some indication of potentially beneficial therapeutic outcomes resulting from strategically combining drugs. DRI results (Figures 4.2 and 4.3) reflect the characteristics of the combinations and our studies suggest that the combinations tested in the androgen-independent cell lines show synergism and this synergism was reflected by the DRI which indicated that substantially less drug is required when using the combination to achieve a therapeutic effect comparable to that achieved with the agents used alone. These results are consistent with the *in*

vivo data shown in Figure 4.4, where it can be suggested that combinations of the ginsenosides and docetaxel at 10 mg/kg were as effective as docetaxel used as a single agent at dose which were 60 to 80% higher. These improvements in *in vivo* activity were not associated with increases in gross toxicity. The results summarized here are in agreement with Wang et al. who showed that docetaxel administered at a dose of 15 mg/kg in nude mice did not induce tumor regression; however complete tumor growth inhibition was possible when using combinations of docetaxel and 25-OH-aPPD (Wang *et al*, 2008).

In an attempt to gain a better understanding of the combination effects that led to improved therapeutic effects as judged by changes in tumor growth rates and associated tumor regress, tumors isolated from animals at the end of the study were assessed for apoptosis and tumor cell proliferation. The apoptotic index, determined from immunohistochemical analysis was higher in tumors from animals treated with docetaxel when compared to tumors from animals in the control, the Rh2+docetaxel and the aPPD+docetaxel treated animals. The reason for why combinations of Rh2 or aPPD with docetaxel appear to interfere with the single agent taxane activity that induces apoptosis is puzzling. Further, Wang et al. have shown that ginsenosides are strong promoters of apoptosis in LNCaP androgen-sensitive and PC-3 androgen-insensitive prostate cancer cells *in vitro* (Wang *et al*, 2008). These data are consistent with those shown in Figure 4.5B, where Rh2 and aPPD treatment alone can cause a significant increase in the measured apoptotic index relative to those obtained in tumors from control animals. Although docetaxel, as a single agent administered at 10 mg/kg, engendered the largest increase (relative to controls) in the measured apoptotic index, this dose failed to induce tumor regression (Figure 4.4D). Combinations of Rh2+docetaxel and aPPD+docetaxel, on the other hand, exhibited lower apoptotic indices but induced significant tumor regression. It is notable

that combinations of Rh2+docetaxel and aPPD+docetaxel significantly inhibited cell proliferation as measured by Ki-67 labeling and the extent of suppression was significantly lower than what was observed in tumors isolated from animals treated with docetaxel alone (Figure 4.5C). Thus the combined activity of these agents may be mediated through mechanisms that cause a decrease in cell proliferation rates as apposed to increases in apoptosis.

In summary, the results presented here provide support for our efforts to develop Rh2 and aPPD for clinical evaluation in combination with docetaxel for treatment of relapsed and hormone insensitive metastatic prostate cancer. Future studies evaluating the combination of ginsenosides with docetaxel are planned in order to further elucidate the nature of the interaction between Rh2 or aPPD with docetaxel that result in synergy.

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Table 4.1. IC_{50} (μM) values following a 72-hour incubation (n = 3). Values are reported as mean ± SEM.

Compound	PC-3	LNCaP	DU145	C4-2
Rh2	12.6 ± 0.21	12.8 ± 1.22	6.6 ± 0.27	7.15 ± 0.41
aPPD	11.9 ± 0.18	12.6 ± 1.66	5.6 ± 0.40	5.6 ± 0.52
Docetaxel	0.15 ± 0.03	0.017 ± 0.002	0.25 ± 0.08	0.048 ± 0.007

Figure Legends

Figure 4.1. *In vitro* drug combination studies analyzed using the combination index (CI) method in four prostate cancer cell lines: PC-3 (A), LNCaP (B), DU145 (C), and C4-2 (D). Rh2+Docetaxel and aPPD+Docetaxel were combined according to the constant ratio experimental design. Dotted line at CI = 1 indicates an additive effect. CI < 1 and CI > 1 indicate synergism and antagonism respectively. Mean value ± SEM is shown with error bars. n = 3.

Figure 4.2. Drug Reduction Index (DRI) expressed as % reduction in IC_{50} for Rh2 and aPPD in combination with docetaxel, compared to their individual IC_{50}s. *In vitro* drug combination studies in four prostate cancer cell lines: PC-3 (A), LNCaP (B), DU145 (C), and C4-2 (D) according to the constant ratio experimental design. Mean value ± SEM is shown with error bars. n = 3.

Figure 4.3. Drug Reduction Index (DRI) expressed as % reduction in IC_{50} for docetaxel in combination with Rh2 or aPPD, compared to its individual IC_{50}s. *In vitro* drug combination studies in four prostate cancer cell lines: PC-3 (A), LNCaP (B), DU145 (C), and C4-2 (D) according to a constant ratio design. Mean value ± SEM is shown with error bars. n = 3.

Figure 4.4. (A-B): *In vivo* docetaxel dose response curve in PC-3 bearing nude mice administered intravenously via the lateral tail vein using a Q7D x 4 dose schedule: 10 mg/kg (filled square); 12 mg/kg (empty square); 14 mg/kg (filled circle); 16 mg/kg (empty circle); 18 mg/kg (filled triangle); 20 mg/kg (empty triangle); and saline (filled lozenge). (A) Change in

tumor volume was followed over time. Average tumor volumes are expressed as a percentage of the average initial tumor volume of each group at day 14, post PC-3 cell inoculation. On day 39, a statistically significant difference was found between the 10 mg/kg treatment group and the 20 mg/kg (p=0.0006) and saline (p=0.0001) groups. All docetaxel were statistically significantly different from saline. (B) *In vivo* toxicity as assessed by decrease in mean body weight. Body weight loss of > 20% is considered severe enough to warrant termination of the animals. Only animals administered the 20 mg/kg dose showed an average weight loss of more than 20% of initial weight at the end of the study. n = 10, mean value ± SEM is shown with error bars. **(C-E):** (C) *In vivo* combination study in PC-3 bearing nude mice. (A) Change in tumor volume was followed over time for animals treated with the oral control (filled triangle, ethanol:propylene glycol:water (2:7:1)), saline (empty triangle), Rh2 (120 mg/kg QD x 5 per week; empty square), aPPD (70 mg/kg QD x 5 per week; filled square), docetaxel (10mg/kg Q7D x 4, *x*), Rh2+docetaxel (Rh2 – 120 mg/kg QD x 5 per week; docetaxel – 10mg/kg Q7D x 4; empty circle), and aPPD+docetaxel (aPPD – 70 mg/kg QD x 5 per week; docetaxel – 10mg/kg Q7D x 4; filled circle). Average tumor volumes are expressed as a percentage of the average initial tumor volume of each group at day 14, post PC-3 cells inoculation. On day 39, a statistically significant difference was found between Rh2 and the controls (p=0.0071 (oral control) and p=0.0007 (saline)), aPPD and the controls (p=0.0215 (oral control) and p=0.0057(saline)), and docetaxel and the controls (p=0.0001 (oral control) and p=0.0001 (saline)). Docetaxel was also statistically significantly different from Rh2 (p=0.0007) and aPPD (p=0.0003). Rh2+docetaxel and aPPD+docetaxel were both statistically significantly different from Rh2, aPPD, oral control, and saline. A statistically significant difference was also found between Rh2 and aPPD (p=0.0412) and between Rh2+docetaxel and aPPD+docetaxel groups. n = 8 for Rh2, aPPD,

docetaxel, Rh2+docetaxel, and aPPD+docetaxel. n = 7 for the oral control and saline. Mean value ± SEM is shown with error bars. (D) % initial tumor volume comparison of the three docetaxel groups: docetaxel alone, Rh2+docetaxel, and aPPD+docetaxel. Tumor regression was achieved only by Rh2+docetaxel and aPPD+docetaxel. The dotted line indicates tumor mass at the start of the study. (E) *In vivo* toxicity as assessed by decrease in mean body weight. No animals showed any signs of toxicity other then weight loss in these studies. Docetaxel, Rh2+docetaxel, and aPPD+docetaxel showed weight loss of less than 20% of initial weight. Mean value ± SEM is shown with error bars. * signifies statistically significant difference from the control.

Figure 4.5.

(A) Representative tissue microarray spots for PC-3 tumors: immunostained using a TUNEL assay by enzymatically labeling the free 3'-OH terminal generated on DNA fragments of 180 to 200 bp. Using this method, apoptotic cells could be identified in areas of viable tissue, as indicated by the arrows. (B) Apoptotic Index summarized from ApopTag® staining of tissue microarrays. Four cores of tissue were extracted per tumor and one tumor per animal. n (total number of cores) = 28 for the control and saline and 32 for Rh2 and docetaxel. On day 39, docetaxel was statistically significantly different from Rh2+docetaxel (p=0.0074) but not from aPPD+docetaxel (p=0.1156). Apoptotic indices of all groups were statistically different from the control. (C) Summarized Ki-67 labeling of tissue microarrays. Mean scores were determined as a percentage of the total number of cells. Four cores of tissue were extracted per tumor and one tumor per animal. n (total number of cores) = 28 for the control and saline and 32 for Rh2 and docetaxel. Mean value ± SEM is shown with error bars. * signifies statistically significant difference from the control. Doc: docetaxel

Figure 4.1

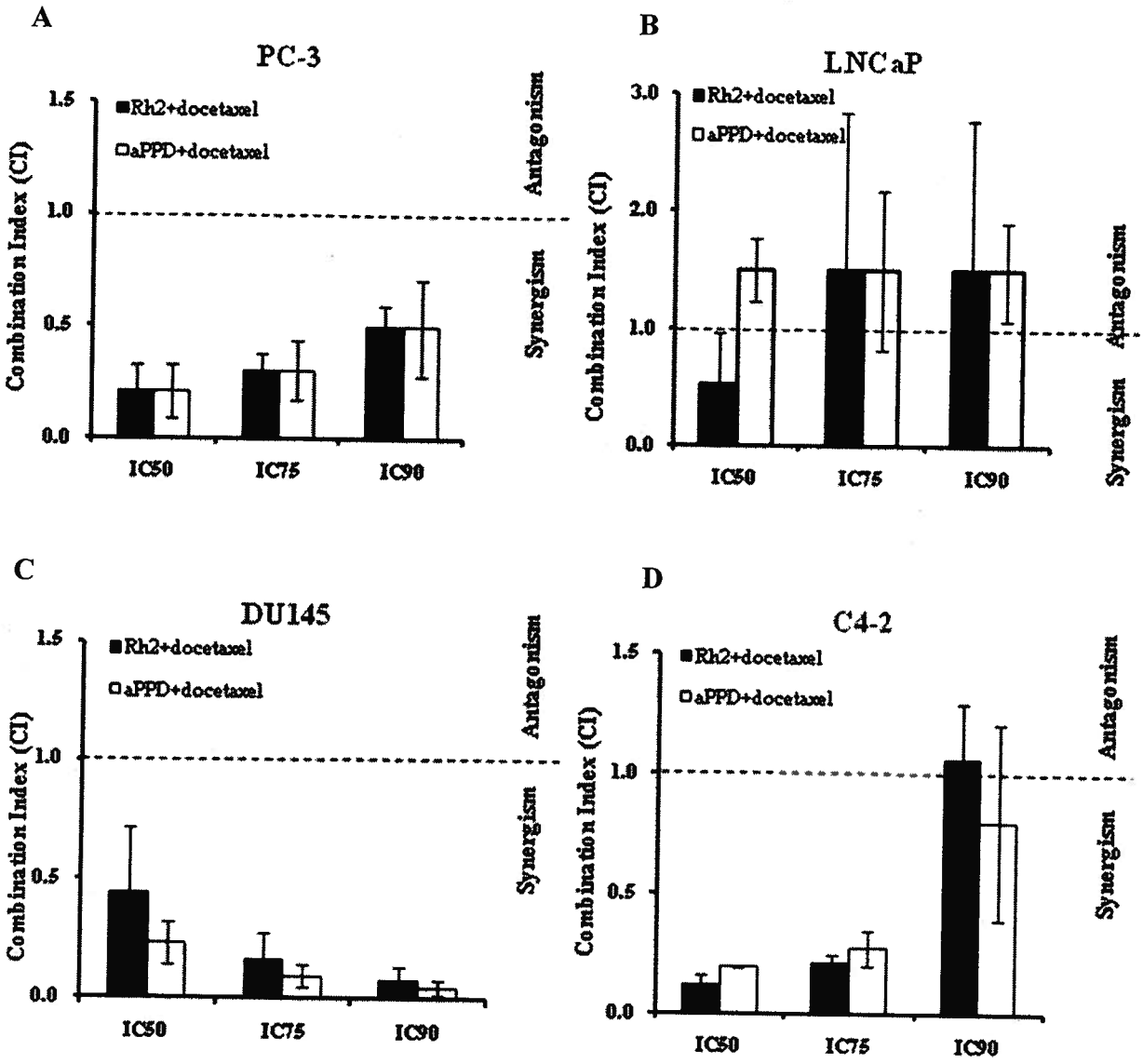

A
PC-3
Rh2+docetaxel
aPPD+docetaxel
Combination Index (CI)
1.5
1.0
0.5
0.0
IC50
IC75
IC90
Antagonism
Synergism
B
LNCaP
Rh2+docetaxel
aPPD+docetaxel
Combination Index (CI)
3.0
2.0
1.0
0.0
IC50
IC75
IC90
Antagonism
Synergism
C
DU145
Rh2+docetaxel
aPPD+docetaxel
Combination Index (CI)
1.5
1.0
0.5
0.0
IC50
IC75
IC90
Antagonism
Synergism
D
C4-2
Rh2+docetaxel
aPPD+docetaxel
Combination Index (CI)
1.5
1.0
0.5
0.0
IC50
IC75
IC90
Antagonism
Synergism

Figure 4.2

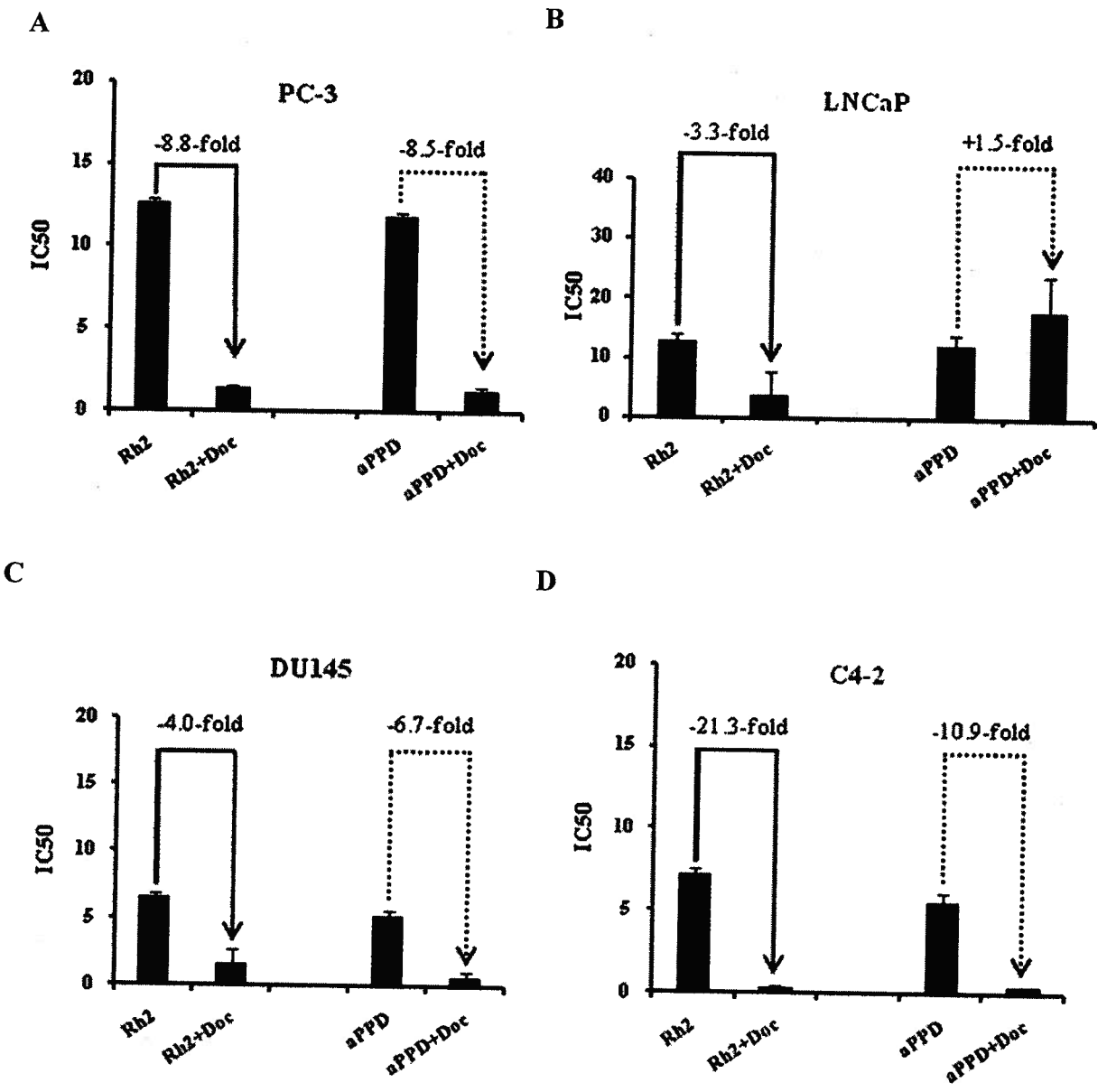

A
PC-3
-8.8-fold
-8.5-fold
IC50
20
15
10
5
0
Rh2
Rh2+Doc
aPPD
aPPD+Doc
B
LNCaP
-3.3-fold
+1.5-fold
IC50
40
30
20
10
0
Rh2
Rh2+Doc
aPPD
aPPD+Doc
C
DU145
-4.0-fold
-6.7-fold
IC50
20
15
10
5
0
Rh2
Rh2+Doc
aPPD
aPPD+Doc
D
C4-2
-21.3-fold
-10.9-fold
IC50
20
15
10
5
0
Rh2
Rh2+Doc
aPPD
aPPD+Doc

Figure 4.3

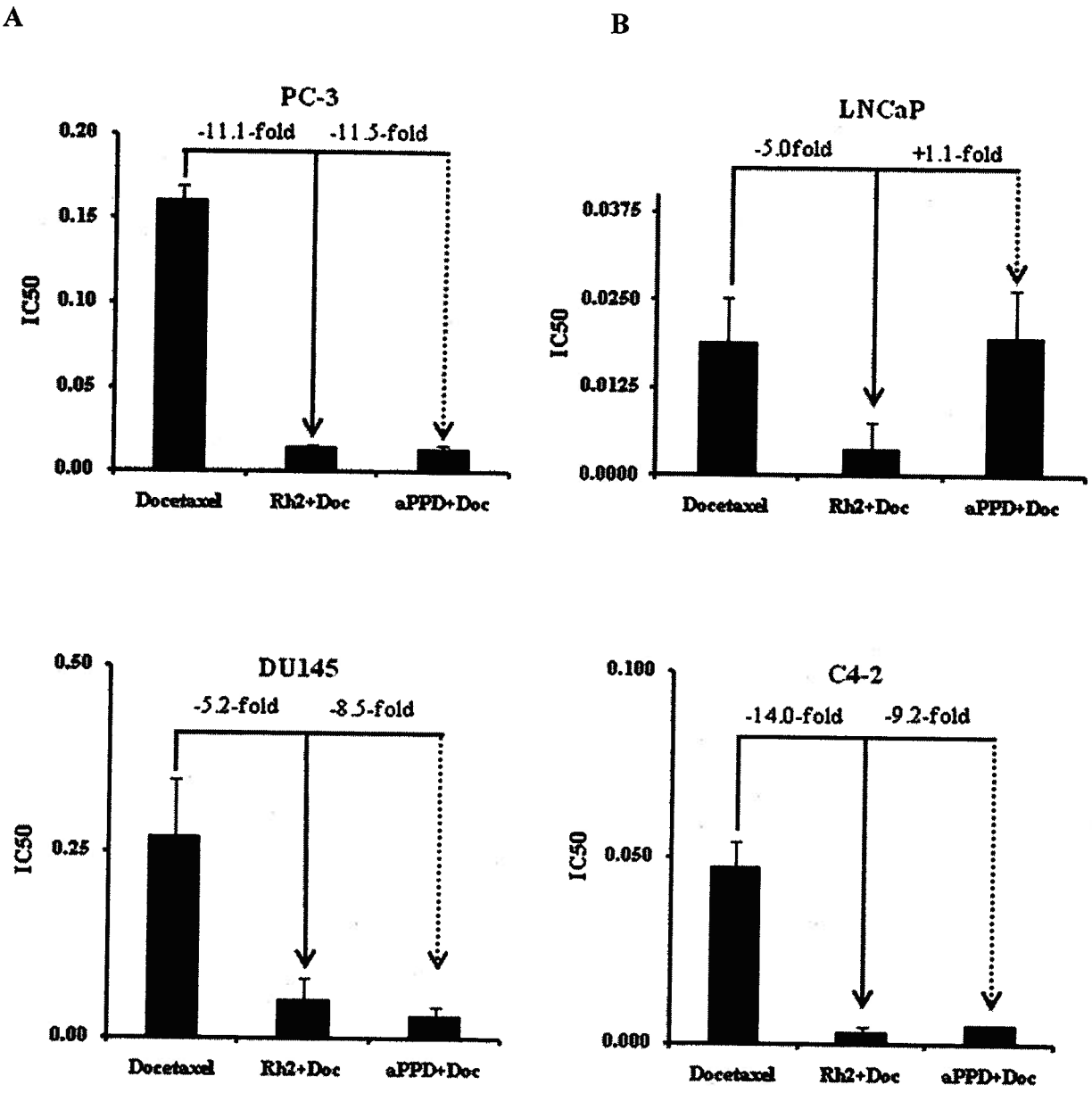

A
B
PC-3
-11.1-fold
-11.5-fold
IC50
0.20
0.15
0.10
0.05
0.00
Docetaxel
Rh2+Doc
aPPD+Doc
LNCaP
-5.0fold
+1.1-fold
0.0375
0.0250
0.0125
0.0000
DU145
-5.2-fold
-8.5-fold
0.50
0.25
0.00
C4-2
-14.0-fold
-9.2-fold
0.100
0.050
0.000

Figure 4.4

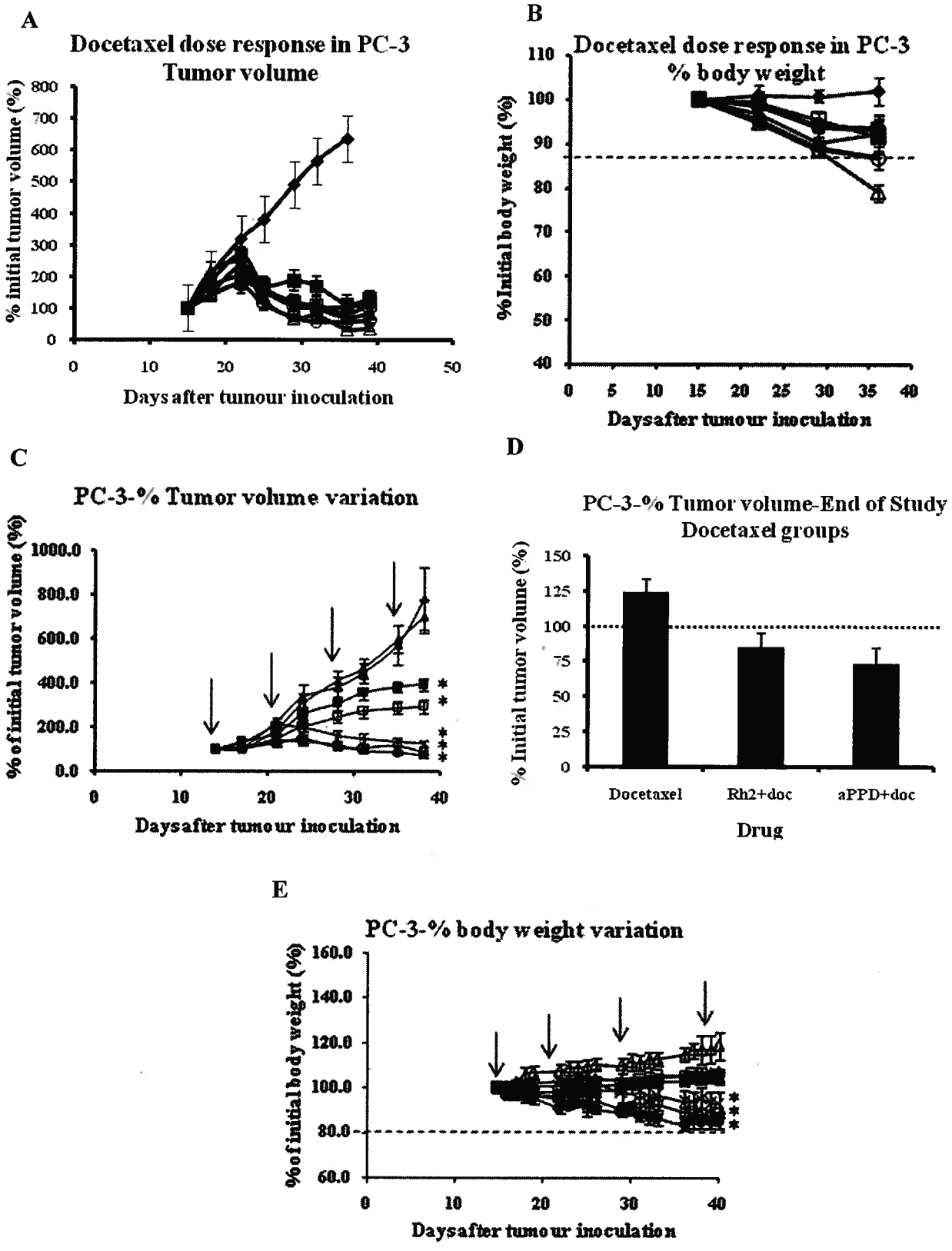
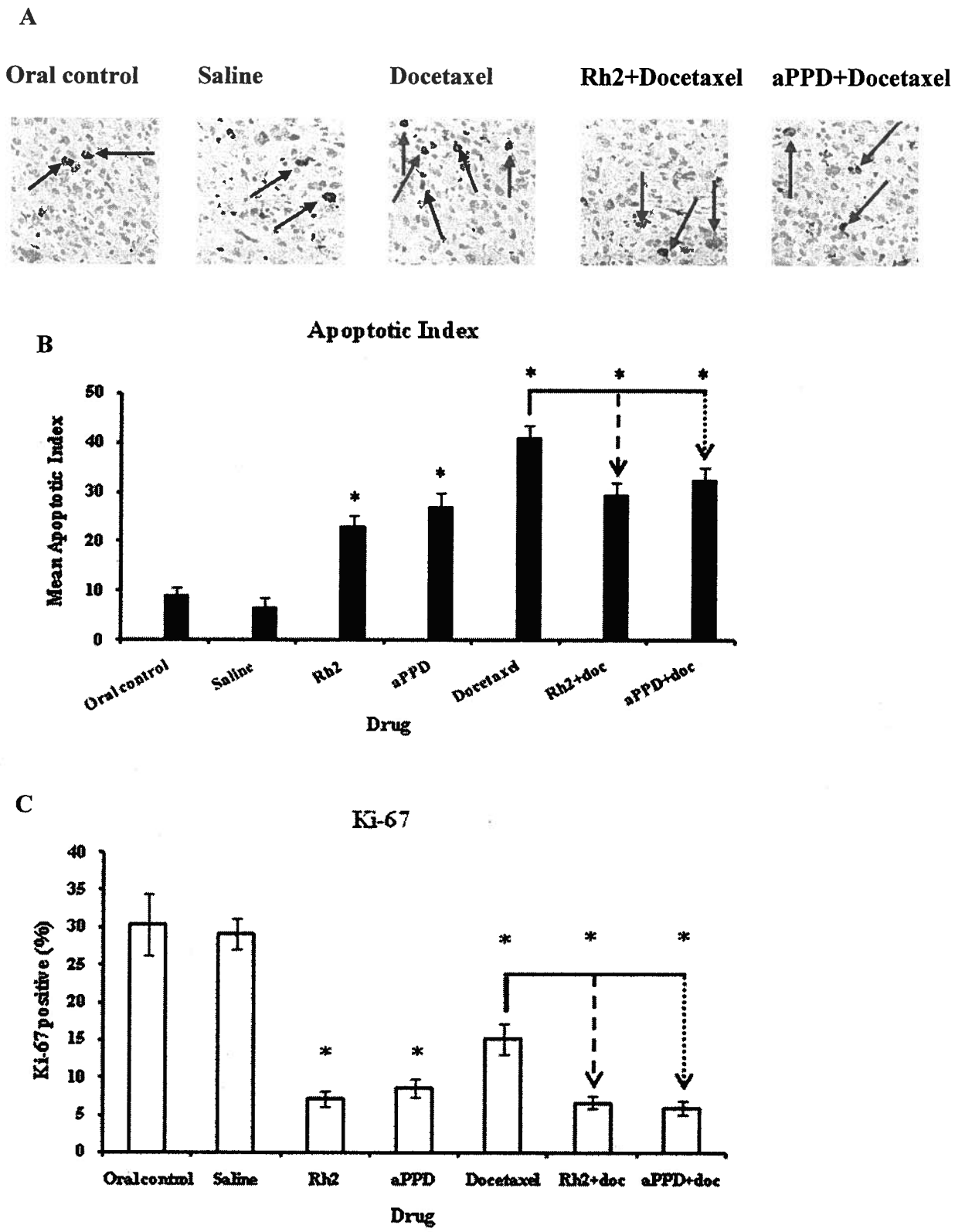

A
Docetaxel dose response in PC-3
Tumor volume
% initial tumor volume (%)
Days after tumour inoculation
B
Docetaxel dose response in PC-3
% body weight
%Initial body weight (%)
Daysafter tumour inoculation
C
PC-3-% Tumor volume variation
% of initial tumor volume (%)
Daysafter tumour inoculation
D
PC-3-% Tumor volume-End of Study
Docetaxel groups
% Initial tumor volume (%)
Docetaxel
Rh2+doc
aPPD+doc
Drug
E
PC-3-% body weight variation
%of initial body weight (%)
Daysafter tumour inoculation

Figure 4.5


A
Oral control
Saline
Docetaxel
Rh2+Docetaxel
aPPD+Docetaxel
B
Apoptotic Index
Mean Apoptotic Index
50
40
30
20
10
0
Oral control
Saline
Rh2
aPPD
Docetaxel
Rh2+doc
aPPD+doc
Drug
C
Ki-67
Ki-67 positive (%)
40
35
30
25
20
15
10
5
0
Oral control
Saline
Rh2
aPPD
Docetaxel
Rh2+doc
aPPD+doc
Drug

5. CONCLUDING CHAPTER

The work presented here follows the integrative, hierarchical approach of drug discovery, providing significant understanding of Rh2 and aPPD at the molecular, cellular, tissue, and *in vivo* levels. At a molecular level, we have demonstrated, for the first time that Rh2 and aPPD both significantly inhibited cell proliferation via Ki-67 inhibition and not following an increase in cellular induction of apoptosis. At a cellular level, we have assessed, for the first time, the characteristics of the *in vitro* drug-drug interactions between Rh2 or aPPD and docetaxel, using the Median Effect Principle and the Constant Ratio combination design, recognized as the most efficient type of combination design (Chou & Talalay, 1984). At the *in vivo* level, we have significantly contributed to the oral formulation and administration of these ginseng metabolites. First we reported the solubility and stability of Rh2 and aPPD in ethanol, for the first time. We have also presented a novel oral gavage formulation, well-tolerated in nude mice, as indicated by the animal percent weight loss and secondary measurements of kidney and liver toxicity. This oral gavage was effective against PC-3 tumor xenografts and allowed for the absorption and biodistribution of Rh2 and aPPD as demonstrated by pharmacokinetic studies. Also for the first time, the *in vivo* combination of Rh2 or aPPD with docetaxel showed that when a lower dose of docetaxel was used (half the maximum tolerated dose), only its combinations with Rh2 or aPPD was effective in not only inhibiting tumor growth but also in inducing tumor reduction. Since the results of these studies have been extensively discussed in each manuscript, I have dedicated the rest of this section for the discussion to expand upon specific aspects of my research in prostate cancer and potential future directions of my work.

Firstly I will touch upon some of the *in vitro* models used for the purpose of my research. While researchers in the field of prostate cancer continue to use the PC-3, DU145, and LNCaP

prostate cancer cell lines, and in doing so, have contributed immeasurably to the understanding of carcinogenesis and cancer progression, notably to the fundamental understanding of growth regulation, cell differentiation, and cell structure and behavior, some intrinsic characteristics of these cell lines still remain controversial. Attempts at isolating and establishing immortalized cell lines as models for studying prostate cancer *in vitro* can be traced back to as early as 1917 (Burrows *et al*, 1917). Burrows et al and the overwhelming majority of subsequent investigators were not successful in this endeavour and recognized the insurmountable challenges at hand, which warranted prostate carcinoma the reputation of being one of the most difficult cell types from which to establish continuous cell lines (Van Bokhoven *et al*, 2003).

Exactly sixty years following the first attempts by Burrows et al., a report of DU145 cell line isolation and establishment was published in 1977 (Mickey *et al*, 1977; Stone *et al*, 1978). DU145 was extracted from the brain metastasis of a 69-year old Caucasian male. The first published report of the establishment of the PC-3 cell line appeared in 1978 (Kaighn *et al*, 1979). PC-3 cells were extracted from a lumbar vertebrae (bone) metastasis of a 62-year old Caucasian male. DU145 and PC-3 are both androgen-independent, do not secrete PSA, and do not express AR (Krueckl *et al*, 2004; Van Bokhoven *et al*, 2003). LNCaP was isolated from the left supra-clavicular lymph node metastasis from a 50-year-old caucasian male and reported in 1980 (Horoszewicz *et al*, 1980; Horoszewicz *et al*, 1983). Unlike DU145 and PC-3, LNCaP is androgen-sensitive, secretes PSA, and expresses AR (Krueckl *et al*, 2004; Van Bokhoven *et al*, 2003).

Further attempts at isolating and establishing immortal prostate cancer cell lines have resulted mostly in failure and in some cases where success was found, the cell lines were later exposed for being derivatives of either DU145, PC-3, LNCaP, or the T24 bladder cancer cell line

(Chen, 1993; MacLeod *et al*, 1999; Pan *et al*, 2001; van Bokhoven *et al*, 2001a; Van Bokhoven *et al*, 2001b; Varella-Garcia *et al*, 2001).

Most investigators involved in the validation of prostatic carcinoma immortal or primary cell lines agree on two critical criteria: (i) the epithelial origin, as established by cytokeratin expression. (ii) The prostatic origin, as established by secretion of PSA, responsiveness to androgens, and expression of AR and DD3 genes (Bussemakers *et al*, 1999; Van Bokhoven *et al*, 2003; Webber *et al*, 1996b; Webber *et al*, 1997). DD3 is a prostate cancer-specific gene overexpressed in the majority of prostate cancers including distant metastases, expressed at a negligible level in nonmalignant prostate tissue, and not expressed in other normal or malignant tissues (Bussemakers *et al*, 1999; Schalken, 2005; Verhaegh *et al*, 2000). Androgens not only regulate prostatic epithelial cell secretion of PSA but also increase AR protein expression (Chen *et al*, 1992; Webber *et al*, 1996a).

Both the PC-3 and DU145 cell lines do not meet the second set of requirements. They are not responsive to androgen, do not secrete PSA and express negligible, if any, AR or DD3 genes. In addition, since the clinical model of progression to androgen independence suggests that the androgen independence stage corresponds to a significant increase in PSA secretion, it is difficult to reconcile and justify the established use of PC-3 cells to mimic this stage, albeit not secreting PSA. The two most common explanations in support of the prostatic origin of PC-3 and DU145 are:

(i) The heterogeneous nature of prostate cancer tumor population. Existence of both AR positive and AR negative malignant cells in prostate cancer tumors before androgen withdrawal therapy in human and after castration in rats have been reported (Sadi *et al*, 1991). Since it has been shown that a higher percentage of metastatic cells are found to be positive for AR than the

primary tumor (Brolin *et al*, 1992). It is thus believed that a fraction of cells in all prostate cancer cell lines express AR in early passages and that androgen independent cells become the dominant cell population, following a natural selection process, in medium deficient of androgens (Webber *et al*, 1996b). The counter argument of this explanation would be that this scenario, if valid, would also apply to the LNCaP cell line. Significantly, two more recently established cell lines also isolated from bone metastasis, the MDA PCa 2a and MDA PCa 2b, are characteristically AR positive, androgen independent but responsive, secrete PSA and express the DD3 gene (Navone *et al*, 1997; Van Bokhoven *et al*, 2003). In my opinion, when prostate cancer cells misplay a critical characteristic such as AR expression, investigators should refrain from using it since AR plays a central role in prostate cancer tumorigenesis and treatment. I would offer that the MDA PCa 2a and MDA PCa 2b cell lines would be best fitted to mimic the androgen-independent stage of prostate cancer. Reproducibility being one of the main principles of the scientific method, it is rather surprising that in spite of recent technological advances in the last 28 years, no investigator has been able to reproduce the successes that struck between 1977-1980, in isolating cell lines that can be uniquely identified as genetically and biologically similar to the PC-3 and DU145 cell lines, without deriving from them.

(ii) Histological evaluation. Some investigators concur that histological similarities between PC-3 and DU145 tumors grown in nude mice and parent tumors are compelling enough to confirm their prostatic origin (Kaighn *et al*, 1979; Webber *et al*, 1997). While I do not dispute the histological value of these cells, I consider the biological requirements just as valuable for a complete and multidimensional understanding of prostate cancer, especially considering that LNCaP cells meet both the histological and biological requirements. A strong case can also be made about LNCaP considering that when inoculated in nude mice, tumors are developed

preferentially in male mice compared to female mice, confirming their androgen responsiveness and prostatic origin (Gleave *et al*, 1992). It is also possible that PC-3 and DU145 may have been established from a rare case where the patient had metastatic carcinoma with very low PSA levels and minimal androgen responsiveness. In this case, it would be more advisable to researchers to investigate scenarios which occur in the majority of cases rather than in exceptional circumstances. I am of the opinion that using PC-3 or DU145 provides an incomplete understanding of prostate cancer, which may be misleading when extrapolated or generalized. I share the view of Van Bokhoven et al. who after cataloging and validating 17 genuine prostate carcinoma cell lines, offer that the heterogeneity of these cells should preclude easy categorization of carcinoma cell lines or their linkage with specific stages of prostate cancer (Van Bokhoven *et al*, 2003).

Validation issues pertaining to PC-3 and DU145 raised here are exacerbated by other problems inherent to the general use of immortalized cell line. Although immortalized cell lines offer the advantage of having an infinite lifespan, which makes it possible to use standardized cell models for research and that *in vitro* systems allow us to select and pinpoint specific mechanisms in a cell, overpassaging of cells engenders selectivity, decreased homogeneity and alteration of key cellular gene functions, in addition to increased potential of cross-contamination (as reviewed by Hughes *et al*, 2007). It has been shown that LNCaP cells with passage of approximately 80, display increased proliferative response to androgens and lower secretion of PSA, compared to cells at passage numbers between 24 and 32 (Esquenet *et al*, 1997). The worth, accuracy, pertinence, consistency, and reproducibility of data generated using these cell lines rest heavily on the type and quality of cell line used. I am of the opinion that the limitations discussed above contribute to contradictory findings in prostate cancer research (i.e.

presence/absence of AR in PC-3 and DU145 cells), ultimately sometimes contributing to confusion rather than progress.

Although the validity of the extrapolation of *in vitro* immortalized cell line investigations to the *in vivo* human situations in drug discovery is often questioned, its impact and influence in recent years can never be underestimated. Some researchers in the field of pharmacology believe that the traditional chemistry and pharmaceutics-based drug discovery approach has been progressively replaced by the discovery of "drugable genome targets" approach, involving molecular biology (Williams, 2005), with a substantial use of immortalized cell culture investigation. It is estimated that in 1994, only 11% of pharmacology fellowships funded by the National Institute of General Medical Sciences at the National Institute of Health (NIH) were awarded to animal research, in comparison to cell culture work (Jobe *et al*, 1994). It is believed that many pharmacology departments in the US did not have access to funding unless their grant applications contained a molecular biology component (Williams, 2005). It could be predicted that the same challenges would be present when a manuscript without a molecular biology component is submitted for publication in prominent journals. These trends prompted the renaming of some pharmacology departments to incorporate the word "molecular" in their titles (In Vivo Pharmacology Training Group, 2002). Some investigators have raised concerns about the target-centered drug discovery trend using largely *in vitro* approaches, including that in this process drug targets are increasingly viewed as static rather than dynamic entities, which they contend, is less reflective of the disease state (as reviewed by Edwards *et al*, 2000; Goldman, 2005). The new approach is also considered overly simplified, reductionist, and exclusionary. They point out that in the case of 7TM receptors, key accessory proteins are frequently absent in transfected cell systems. These proteins may significantly alter the receptor function and the

recognition characteristics of novel ligands. This example is believed to be one of the reasons why New Chemical Entities (NCEs) identified in engineered cell systems are required to be reassessed for activity in systems where the receptor/target occurs naturally (Kenakin, 1996; Maudsley *et al*, 2005).

In the past decade only, the target-centered approach of drug discovery has produced at least 518 and 1300 potential drug targets for protein kinases and phosphatases only, respectively (Manning *et al*, 2002). This is a remarkable achievement considering that the number of protein kinase drug targets is equivalent to the approximately 500 distinct drug targets established over centuries and catalogued in the current pharmacopoeia (Drews, 1996). Concerned drug discovery investigators acknowledge and appreciate this accomplishment and have no issue over the increase in their funding but deplore the fact that the funding process is conducted with the exclusion of more traditional integrative research approaches which include animal models. They believe that this exclusionary approach in funding is largely responsible for the current drought of new drugs (Duyk, 2003; Milne, 2003) and the transformation of the pharmaceutical industry to a less successful business compared to the 1970s to mid 1980s when numerous successful drugs were discovered, including the chemorherapeutics cisplatin and taxol (Goozner, 2004; Sneader, 1985).

Considering that immortalized prostate cancer cell lines currently in use are not representative of primary adenocarcinoma of the prostate and are largely susceptible of genetic and biological alterations and cross-contamination, in addition to the controversial characteristics associated with PC-3 and DU145, my suggestion would be that biomedical researchers should focus their efforts primarily in the development of efficient and successful ways to extract and grow primary cultures of malignant prostatic cells and their normal epithelial counterparts, for

comparison. Growing primary cultures has been rendered less challenging thanks to recent technological advances in that field (as reviewed by Peehl & Sellers, 2002).

Contrary to the impression that might be conveyed by my discussion on "target-centered" biomedical approach, I advocate greater use of *in vitro* research and would discourage the use of laboratory animals unless indispensable. Despite the invaluable contribution of laboratory animals in pre-clinical research, only 20% of drugs successful in pre-clinical trials are approved by the FDA (FDA, 2008). The very modest human predictability and reliability of animal models is mostly due to significant genetic differences between species. Even the Chimpanzee, our closest evolutionary relative, displays a difference in approximately 80% of protein expression despite a mere 1 to 2% nucleotide difference between our the two species (Glazko *et al*, 2005). In addition, many of the proteins expressed and characterized as being equivalent to those expressed in the human also share only very modest sequence homology between species and consequently have quite different substrate specificities and/or fundamental functionalities (Nelson, Pharmacogenetics, 1996). Rodents that are used routinely for toxicity studies are even further disparate from the human, genetically and phenotypically. It also has been shown that the immune system of rodents is greatly affected and altered by the handling, restraint and related stress of testing procedures, possibly distorting both disease progression and response to test compounds (Balcombe *et al*, 2004; as reviewed by Knight *et al*, 2006). Genetic and phenotypic differences between species often translate to significant differences in disease progression, absorption, tissue distribution, metabolism and excretion, toxicity, and efficacy (as review by Bailey, 2005; Knight, 2007). In addition, financial costs of maintaining and experimenting on animal are also considerable. It is estimated that the maintenance of one

laboratory Chimpanzee in captivity costs between $300,000 U.S. to $450,000 U.S. for a life span (Cohen, 2007b).

Legislations intended in prohibiting the use of Chimpanzees in invasive research and breeding moratoriums in the US (Cohen, 2007a; HSUS, 2008) and Europe (British Psychological APC, 1998; Society, 2002) have accentuated efforts in developing volunteer studies, *in vitro* and physicochemical techniques, and computer modeling intended for replacing animal procedures. A report entitled *The Statistics of Scientific Procedures on Living Animal* issued by the British Home Office concluded that the British industry in general has significantly reduced its use of animal testing, compared to academia where the practice has increased by 52% (The Stationery Office, 2005). Langley et al. report that the European Centre for the Validation of Alternative Methods (ECVAM), created in 1991, has successfully validated more than 18 full or partial animal testing replacement methods, eight of which have already gained regulatory acceptance in the industry (as reviewed by Langley *et al*, 2007). Three examples of animal testing replacement are often cited as significant achievements: (i) the rat skin and human skin model assays and the Corrositex model, used for testing chemical corrosivity, thus replacing severe *in vivo* tests on rabbits. (ii) The cell-based neutral red uptake assay for photo-irritancy has replaced inconvenient tests on mice. (iii) A method of assessing skin penetration of drugs and pesticides has replaced testing on rodents (as reviewed by Langley *et al*, 2007). Although encouraging, these modest accomplishments are far from capturing the complexity of *in vivo* organisms and of providing comparable of greater human predictability than animal models currently used, particularly those pertaining to cancer and carcinogenesis.

In summary, considering the cost and poor human predictability associated with animal models, in addition to ethical concerns, biomedical researchers should be encouraged to develop

in vitro models for the identification of drug targets and assessment of drug toxicity and efficacy. These models should be able to capture, to a significant extent, the complexity exhibited by living organisms and avoid being over simplistic and far removed from reality. Funding agencies need to be more progressive yet retain the ability to fund and encourage old approaches to drug discovery that have been proven successful and not discriminate against them, for the benefit of trendy but yet unproven new approaches.

5.1 Future directions

Since we have shown that *in vitro* combination of Rh2 or aPPD with docetaxel yields synergistic outcomes against PC-3 cells, it would be useful to determine and compare the cellular uptake of these agents alone and in combination. This investigation would involve two steps: (i) cellular uptake kinetics and (ii) inhibition of efflux transporters.

For cellular uptake kinetics studies, PC-3 cells would be treated with solutions of Rh2, aPPD, or docetaxel, alone and in combination and incubated at 37°C for 15, 30, 45, 1 h, 3 h, 6h, 12h, 24h, 48 h, and 72 h. At the end of the incubation period, the solution will be aspirated, cells washed, excised, and the concentration of the uptaken drugs determined by analytical HPLC and MS methods.

While it is known that P-gp efflux transporters are expressed at relatively high levels in PC-3 cells (Theyer *et al*, 1993), it is also established that docetaxel is a P-gp substrate and that one of the important mechanisms of docetaxel resistance is an overexpression of P-gp in cancer cells (Bissery *et al*, 1995; Shirakawa *et al*, 1999; van Ark-Otte *et al*, 1998). On the other hand, as reported earlier, Rh2 and aPPD have been shown to significantly enhance the cytotoxicity of mitoxantrone in human breast carcinoma MCF-7/MX cells which overexpress breast cancer

resistance protein (BCRP). Their effect was attributed to the inhibition of mitoxantrone efflux and enhanced mitoxantrone uptake (Jin *et al*, 2006). Thus one aim of the inhibition of efflux transporter experiments would be to investigate the ability of Rh2 and PPD in inhibiting P-gp efflux transporters in PC-3 cells. Cells will be treated and incubated with various concentrations of docetaxel in the absence or presence of Rh2 or aPPD for the optimum time period as determined by the uptake study. MS-209, an established P-gp inhibitor could potentially be used as the positive control compound. MS-209 is a quinolone derivate currently in clinical trials for breast and lung cancer treatments (Narasaki *et al*, 1997; Perez-Tomas, 2006; Robert & Jarry, 2003). At the end of the incubation period, the solution will be aspirated, cells washed, excised, and the concentration of the uptaken drugs determined by analytical HPLC and MS methods.

The multidrug resistance 1 gene (MDR1), which encodes the P-gp protein, is expressed in several tissues including the kidney, colon, liver, lung, brain, and the prostate (Fojo *et al*, 1987). Should the experiments presented above conclusively show that Rh2 and aPPD increase the cellular uptake of docetaxel, *in vivo* administration of Rh2 or aPPD in combination with docetaxel will be carried out as part of a pharmacokinetic experiment. The aim of this experiment would be to evaluate alteration in tissue absorption of docetaxel when coadministered with Rh2 and aPPD. Here, mice would be administered Rh2, aPPD, docetaxel+Rh2, docetaxel+aPPD, or docetaxel+ MS-209. Tissues would be excised at several time points, and the drugs extracted and quantitated using analytical HPLC and MS methods.

In summary, in combining the results from the experiments outlined above, we would demonstrate whether the cellular uptake of docetaxel by PC-3 cells *in vitro* and *in vivo* is enhanced via inhibition of P-gp transporters by Rh2 and aPPD and that this mechanism plays a significant role in the *in vivo* and *in vitro* synergy that we showed in our third manuscript.

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APPENDIX A: First Manuscript Chapter – Title page information

Pre-clinical evaluation of Rh2 in PC-3 human xenograft model for prostate cancer *in vivo*: Formulation, Pharmacokinetics, Biodistribution and Efficacy

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Competing interest statement: The authors declare that they have no competing financial interests

Running title: Pharmacokinetics and Efficacy of Rh2
Key words: Ginsenoside Rh2, prostate cancer, pharmacokinetics, biodistribution, efficacy

APPENDIX B: Second manuscript chapter – Title page information

A novel oral dosage formulation of the ginsenoside aglycone aPPD exhibits therapeutic activity against a hormone insensitive model of prostate cancer

Alain G. Musende[1,2], Andy Eberding[1], Catherine Wood[1], Hans Adomat[1], Ladan Fazli[1], Antonio Hurtado-Coll[1], William Jia[3,4], Marcel B. Bally[2,5,6], and Emma Tomlinson Guns[1,6,7].

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Runninghead: Efficacy of aPPD against the PC-3 prostate model

Key words: Ginsenoside, prostate cancer, pharmacokinetics, biodistribution, efficacy

APPENDIX C: Third manuscript chapter – Title page information

Rh2 or its aglycone aPPD in Combination Therapy with docetaxel

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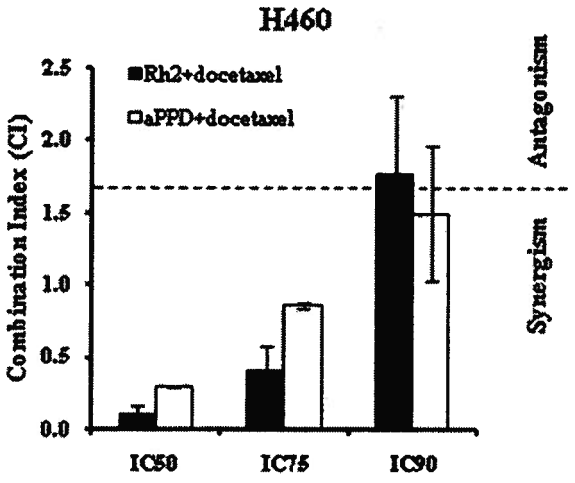
Competing interests statement: The authors declare that they have no competing financial interests

Running title: Combination of Rh2 and aPPD with docetaxel

APPENDIX D: (I) *In vitro* drug combination studies analyzed using the combination index (CI) method in H460 non-small-cell lung cancer cell line. Rh2+Docetaxel and aPPD+Docetaxel were combined according to a constant ratio design. Dotted line at CI = 1 indicates additive effect. CI < 1 and CI > 1 indicate synergism and antagonism respectively. n = 3. (II and III) Drug Reduction Index (DRI) expressed as % reduction in IC_{50} for Rh2 and aPPD in combination with docetaxel, compared to their individual IC_{50}s. n = 3.

I



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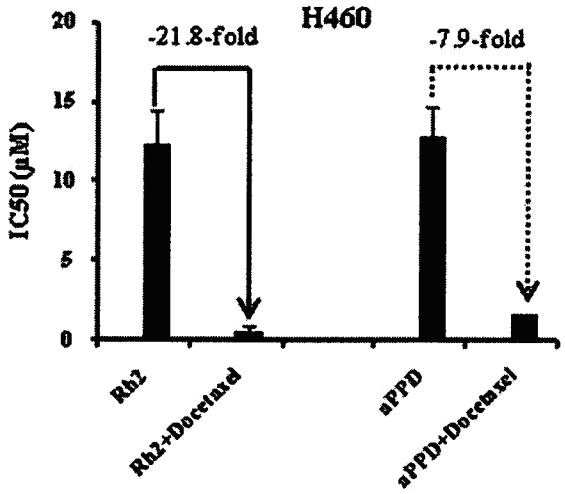
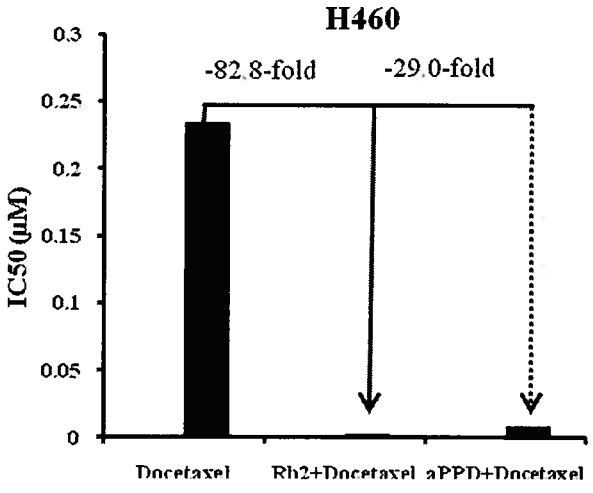
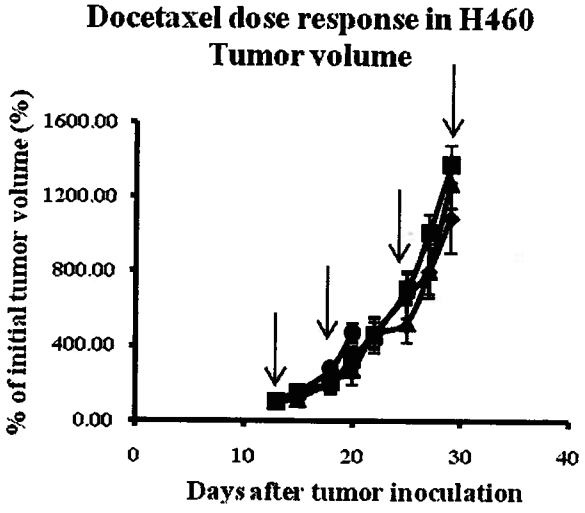


III



APPENDIX E: *In vivo* docetaxel dose response study in H460 non-small-cell lung cancer cell bearing nude mice administered intravenously via the lateral tail vein using a Q7D x 4 dose schedule: 10 mg/kg (filled diamond); 15 mg/kg (filled square); 20 mg/kg (filled triangle); and saline (filled circle). The mice were monitored daily for changes in weight and other signs of acute toxicity. (I) Change in tumor volume was followed over time. Average tumor volumes are expressed as a percentage of the average initial tumor volume of each group at day 14, post H460 cell inoculation. The control group mice reached a tumor volume of 1000 mm^3 by day 20, warranting termination of the animals. Volumes were calculated using $V = (LxWxH)/(\pi/6)$. Mean value ± SEM is shown with error bars. (II) *In vivo* toxicity as assessed by decrease in mean body weight. Body weight loss of > 20% is considered severe enough to warrant termination of the animals. No animal showed an average weight loss of more than 20% of initial weight at the end of the study. * signifies statistically significant difference from the control. Mean value ± SEM is shown with error bars. n = 10.

A



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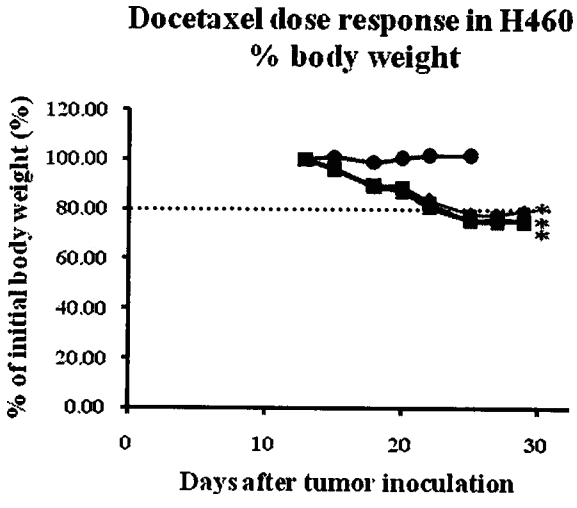

APPENDIX F: *In vivo* efficacy of Rh2 in H460 non-small-cell lung cancer cell bearing nude mice. Changes in tumor volume were followed over time for animals treated with the oral gavage vehicle control (ethanol:propylene glycol:water (2:7:1), filled square), saline (empty square), Rh2 (120 mg/kg QD x 5, filled triangle), aPPD (70 mg/kg QD x 5, empty triangle) and docetaxel (20mg/kg Q4D x 4, filled circle). Average tumor volumes are expressed as a percentage of the average initial tumor volume of each group at day 14, post H460 cell inoculation. Volumes were calculated using V = (LxWxH)/(π/6). Mean value ± SEM is shown with error bars. n = 10